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Defining Neoepitopes that Contribute to Tumor Immunity

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Over the last half century, it has become well established that cancer can elicit a host immune response that can target the tumor with high specificity. Only within the last decade, with the advances in gene sequencing and bioinformatics approaches, are we now on the forefront of harnessing the host's immune system to fight cancer. Recently, many great strides have been taken towards understanding effective tumor specific major histocompatibility complex I restricted epitopes, or neoepitopes. However, many fundamental questions still remain that must be addressed before this knowledge can live up to its full potential in the clinic. Issues ranging from the accurate identification all the somatic mutations in a given tumor sample to the prediction of the "best" neoepitopes and translation of that information into the most effective treatment illustrate the hurdles that remain. In this thesis, I present results of an exhaustive and exhausting study where I have tested hundreds of long peptides each containing a single nucleotide variant (SNV) (with the mutation in the center of the peptide) for their ability to elicit tumor rejection and independently, $CD8^+$ T cell response. I observe that (i) about 7% of all SNVs lead to generation of peptides that can mediate tumor rejection to any significant degree; (ii) each peptide alone elicits a modest protection, and a combination elicits stronger protective immunity; (iii) most neoepitopes that elicit tumor rejection have poor binding affinity for MHC I and have positive values for Differential Agretopic Index; (iv) even though the protective responses are CD8-mediated, there is no correlation between a neoepitope's ability to elicit a measureable CD8 response and tumor rejection; (v) and lastly that the neoepitopes elicit a variety of T effector cell phenotypes and, in a preliminary analysis, one particular CD8 T cell phenotype is a better predictor of tumor rejection. Through ongoing and subsequent future work, the aim is to truly understand what makes a good neoepitope and in turn translate this knowledge into effective treatments.

(Modified from Brennick CA, George MM, Corwin WL, Srivastava PK, Ebrahimi-Nik. Immunotherapy. 2017 Mar, 9(4):361-371. doi 10.2217/imt-2016-0146.)

Defining Neoepitopes that Contribute to Tumor Immunity

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Defining Neoepitopes that Contribute to Tumor Immunity

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I dedicate my work to my grandmother, who always has been my number one supporter. Seeing her struggle in her final days with cancer treatment and her passing has fueled me to make scientific discoveries to find less harmful and more effective treatment modalities against cancer.

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Abbreviations

NK	Natural Killer Cells
DCs	Dendritic Cells
CTL	Cytotoxic T Cell
MHC	Major Histocompatibility Complex
HSP	Heatshock Protein
ER	Endoplasmic Reticulum
TAP	Tansporter associated with Antigen Processing
ERAP	ER aminopeptidases associated with Antigen Processing
TCR	T Cell Receptor
APC	Antigen Presenting Cell
TC	Tumor Challenge
TIL	Tumor Infiltrating Lymphocyte
MS	Mass Spectrometry
ANN	Artificial Neural Network
DAI	Differential Agretopicity Index
Th	T helper
CAR	Chimeric Antigen Receptor
TME	Tumor MicroEnvironment
ACT	Adoptive Cell Transfer
SLP	Synthetic Long Peptides
SNV	Single Nucleotide Variants
BMDC	Bone Marrow derived Dendritic Cells
TCI	Tumor Control Index
MFI	Mean Fluorescence Intensity
SD	Standard Deviation
SEM	Standard Error of the Mean
pMHC	Peptide bound to MHC
Treg	T Regulatory
HPD	HyperProgressive Disease

Chapter 1

Introduction

(Modified from Brennick CA, George MM, Corwin WL, Srivastava PK, Ebrahimi-Nik. Immunotherapy. 2017 Mar, 9(4):361-371. doi 10.2217/imt-2016-0146.)

Introduction

It is now appreciated that cancer is a profoundly patient specific disease, where no two tumors are alike. The foundation of this insight stemmed from the early murine work of Ludwig Gross[1], Prehn and Main[2], and George and Eva Klein[3] that clearly demonstrated the tumor immunogenicity is not only patient specific but inherently specific to the individual tumor itself. Continued cancer research in areas such as immunology, genomics, biochemistry, metabolomics, etc. have further enhanced our appreciation of the vast complexity of this malady.

A critical realization was that of the immense heterogeneity not just between patients diagnosed with the same type of cancer but also within a single patient's lesion(s)[4,5]. This heterogeneity has made conventional cancer therapies limited in their success to treat all cancers due to each tumor's individuality. Further, the concept of immunoediting[6], or the selective pressure of the immune system to drive tumor evolution, has helped as a framework to understand not only tumor development but also treatment resistance and metastasis. In response to these insights, considerable efforts have begun to develop and adopt "precision medicine" or cancer therapies that aim to treat in a patient specific manner. To this end, a number of cancer immunotherapies have been developed that look to exploit the immune system's exquisite ability to selectively target cancer cells[7–9]. While considerable gains have been made with regard to treatment outcomes for many patients, there still remains a fundamental deficiency in our knowledge of the precise immunological underpinnings behind this tumor specificity observed over a half a century ago.

Since the time it was initially proposed over two decades ago[10], it has now become well established that somatic, or so-called passenger, mutations within the tumor give rise to new epitopes, or neoepitopes. Considerable experimental evidence for this idea was obtained starting in the 1990's (Table 1). Further, evidence supports that neoepitopes are recognized by the adaptive immune system as "mutated self" and serve as the means by which immune systems can differentiate cancer from normal cells[11]. Thus, neoepitopes make strong candidates for personalized cancer immunotherapy vaccines. With the recent advancements in genomic sequencing and bioinformatic approaches, the feasibility of personalized therapy in the clinical setting is now on the forefront. However, there are still major obstacles that need to be addressed. To this day, most of the putative neoepitopes identified have not worked in rejecting tumor, with an average of less than 7.5% of predicted neoepitopes being capable of eliciting a tumor specific immune response[12]. Issues ranging from the accurate identification all the somatic mutations in a given tumor sample to the prediction of the "best" neoepitopes and translation of that information into the most effective treatment illustrate the hurdles that remain. Here, I will discuss these challenges and the work being done to address them in an effort to truly understand what makes a good neoepitope and how best to translate this knowledge into effective treatment modalities.

Table 1 | Tumor-specific antigens of mouse tumors

Gene/Protein	Tumor	Restricting MHC	Peptide[a]	Codon	Elicits protective tumor immunity?	Ref
L9 ribosomal protein	6123A Squamous Ca	IE^k	DFNHINVELSHLGK	47	YES	13
P68 RNA helicase	8101 Squamous Ca	K^b	SNFVFAGI	551	Not testable, since tumor is a regressor	14
P53	Meth A fibrosarcoma	K^d	KYICNSSCM	234	YES	15
ERK2	CMS5 fibrosarcoma	K^d	LQYIHSANVL	136	YES	16
L11 ribsomal protein	Meth A fibrosarcoma	IE^d	EYELRKHNFSDTG	97	YES	17
Akt 5' untranslated region	RLM1 leukemia	L^d	IPGLPLSL	5' untranslated	YES	18,19

[a]The residues modified by the mutation are indicated in red

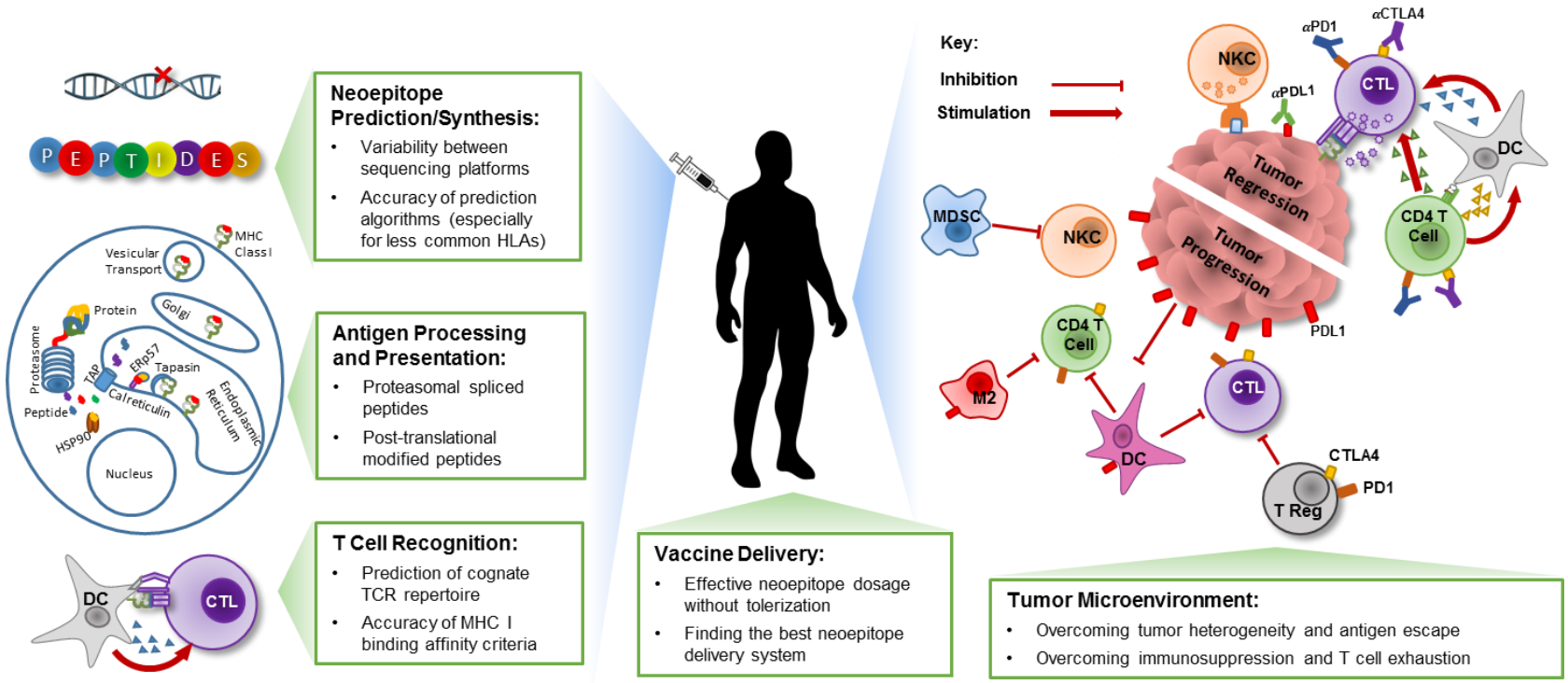


Figure 1 | The current challenges of neoepitope based therapies
Displayed here is a schematic of the major steps involved in neoepitope focused cancer immunotherapies. Each step in this figure illustrates the key challenges that need to be addressed for improved clinical translation. See the text for in depth description.

From ubiquitination to recognition

Cancer immunotherapy

B cells, as well as many other types of immune cells, such as T cells, are included in the use of many immune cell types

cells, natural killer cells (NK) and dendritic cells (DCs). Cytotoxic $CD8^+$ T cells (CTLs) are the most potent cell type which when activated, are able to recognize tumor specific antigens and distinguish between normal and cancerous cells. To achieve this tumor specific killing, cancer vaccines containing MHC class I restricted epitopes should activate $CD8^+$ T cells to specifically kill cancerous cells[20,21]. This involves a number of steps which are summarized as follows[22]. Degradation of ubiquitinated proteins by the proteasome in the cytosol, chaperoning of peptides by heat shock protein (HSP) 90 in the cytosol, active transport into endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) and trimming to appropriate length by ERAP (ER aminopeptidases associated with antigen processing), loading onto the peptide binding cleft of the Class I MHC molecules with the help of chaperone proteins such as tapasin and transport to the cell surface which is then recognized by the T cell receptor (TCR) on $CD8^+$ T cells. Neoepitopes are those peptides that arise from somatic mutations and recognized as different from self and presented by antigen presenting cells (APCs) such as DCs and the tumor cells itself[10]. Here cross presentation plays an important role as the APC is able to translocate exogenous antigens from the phagosome into the cytosol for proteolytic cleavage into MHC I epitopes by the proteasome[23].

Although the binding affinity of a peptide to MHC I is one of the most studied criteria in the search for neoepitopes, many other factors discussed here play an important role in whether a neoepitope is finally presented on the tumor cells and whether it is taken up by APCs and cross presented in order to activate neoepitope specific $CD8^+$ T cells. Thus far, there are no reliable methods to accurately verify that *in silico* identified neoepitopes can actually elicit tumor regression or an immune response. As mentioned earlier, the peptides released by the proteasome

are transported to the ER from the cytosol by TAP. TAPPred is an on-line tool to predict binding affinity of peptides toward the TAP[24]. However, these tools for proteasomal cleavage or TAP binding and transport are limited and not accurate and most neoepitope prediction algorithms do not take into consideration this aspect[25]. Peptide trimming by ERAP is important for the loading of the correct sized peptide onto the peptide binding cleft of MHC I. Thus far, no prediction algorithm accurately incorporates the function of ERAP into predicting Class I restricted neoepitopes.

It has been found, in various solid and hematological tumors, that some of the mutations occur in genes that are involved in antigen presentation. If this phenomenon occurs, then predicting neoepitopes for such tumors and using them for immunotherapy may not result in an immune response or tumor regression because the presentation of neoepitopes on the cell surface is defected. The aberrations in the antigen presentation machinery range from defects in proteasomal subunit patterns, TAP, ERAP, other chaperone proteins and MHC I. They occur individually or in combination, and the frequency and nature of the defects vary according to the type of tumor. Defects in surface expression of MHC I molecules have been demonstrated in a large variety of human tumors[26]. As with the above-mentioned defects in the antigen presentation machinery, particularly loss of MHC I surface expression, the use of neoepitopes that, *in silico,* are predicted to be recognized by the immune system, based on MHC I structure, may not necessarily be presented by the tumor cells. In such cases, immunotherapy with these neoepitopes will not elicit an immune response or tumor regression. The addition of mass spectrometry (MS) may play a key role in prediction pipelines to account for the antigens that are actually presented on the tumor surface. It must be mentioned that this method has not yet

reached the level of being a high through put approach. However, improved sensitivity of MS in identifying tumor derived neoepitopes in patient's tumors validated by neoepitope specific anti-tumor immune response has been recently reported[27].

Another major aspect to eliciting a sufficient T cell response against a neoepitope, is its ability to bind its cognate TCR. It has been clearly demonstrated the TCR affinity and avidity are crucial to the T cell response amounted[28–30]. A great effort has been made to develop programs that can predict the ability of a MHC I with is peptide to bind to a known T cell receptor (TCR). Thus far the programs available are CTLPan and NetCTLPan, but with a limited known TCR peptide MHC I interactions known, these programs are limited in there accuracy[31]. With new technologies emerging for the identification of TCRs to their cognate antigen these programs may become of great use to predict putative neoepitopes.

The development of prediction algorithms or programs that incorporates not only the binding affinity of the neoepitope on MHC I but also the other steps of antigen processing, presentation and TCR recognition remains an important step towards improving the accuracy of predicting successful neoepitopes.

Are we being misguided by binding affinity?

In an effort to better predict putative neoepitopes from the large set of somatic mutations called for a given tumor, the criterion of binding affinity of a neoepitope to its cognate MHC I is applied. The most readily used technique for measuring binding affinity *in vitro* is by measuring the concentration of a given peptide needed to compete with a standardized peptide already bound to a given MHC I allele[32]. This results in an IC_{50}, half maximal inhibitory concentration, score for a given peptide and its cognate MHC I. It is important to note that this technique does

not account for the highly complex peptide loading pathway that has been observed *in vivo*[22], which could aid in the loading of less stable peptides[33]. It has been demonstrated that the peptide loading complex, most importantly tapasin, plays a crucial role in the loading of endogenous peptide, where *in vitro* studies would consider that peptide unable to bind MHC I[34]. With current methodologies unable to recapitulate the peptide loading complex *in vitro,* measuring whether or not a peptide will bind to MHC I is limited to the measurement of the IC_{50} for a given peptide. Furthermore, these *in vitro* measured IC_{50} concentrations, from known viral and model antigens, are used to train current binding affinity predictions algorithms used today.

There are currently more than a dozen various prediction algorithms for predicting the binding affinity of peptides, with NetMHC[35] being the most widely used and validated algorithm for neoepitope prediction pipelines. Like most prediction algorithms, NetMHC has been trained with an artificial neural network (ANN), and outputs a predicted IC_{50}. With over 11,000 variants at the human class I loci identified, according to the IMGT/HLA database[36], and the less common HLA's peptide binding kinetics being poorly studied, accurately predicting a peptides ability to bind to its cognate MHC I has been very difficult in itself. For the more commonly studied MHC I molecules, standard criteria have been established to predict the likely immunogenic peptides that can be applied to all HLAs as more are characterized.

The thresholds used today to determine which peptides are most likely to elicit a cytotoxic T lymphocyte (CTL) response were established over two decades ago, and set the standard that an $IC_{50} \leq 500$ nM, preferably 50 nM or less, is considered a strong to intermediate binder and determines an epitope's capacity to elicit a CTL response[37]. This criterion has been highly

validated in viral and model antigen systems where there is no "self" counterpart. Since neoepitopes arise from altered self-peptides, neoepitope specific T cells potentially face thymic deletion and tolerization. This issue was addressed by Duan *et al.*, where they tested the differential agretopicity index (DAI) score, that compares the binding affinity of the mutated peptide to the reference peptide[38]. This score indirectly accounts for how different the peptide is from "self". In that study, they identified three neoepitopes that were able to effectively inhibit tumor growth in two different BALB/c tumor models, yet had IC_{50} scores significantly above the 500 nM threshold. By current standards, these neoepitopes would have been overlooked and not considered immunogenic based of their binding affinity alone.

On the contrary, studies by Gubin *et al.*[39] and Yadav *et al.*[40] have each identified neoepitopes that fit the strong affinity criterion that were able to effectively target the tumor. It is worth noting that work done by Assarsson *et al.*[41] in a viral model system established that about 7.5% of the predicted high affinity binders are actually recognized by the immune system, yet in the tumor model system the frequency of validated high affinity binders to those predicted to be immunogenic is significantly lower. This observation is supported by the work of Van Rooij *et al.* that reported that less than 1% of the high affinity binders predicted from a melanoma patient were able to be recognized by the patient's T cells[42].

Surprisingly, it was recently found that proteasome-generated spliced epitopes account for one fourth of the HLA class I immunopeptidome in terms of abundance[43]. This accounts for a large repertoire of proteasomal spliced peptides and current prediction methods do not take these peptides into consideration, as criteria for what peptides get spliced has not been established.

Moreover, the importance of post-translationally modified proteome substrates is of importance and currently overlooked. It has been clearly demonstrated that post translational modifications, such as phosphorylation, methylation, and glycosylation, can be recognized by the immune system as foreign compared to their unmodified counterpart[44]. With the advancement of mass spectrometry, it is now possible to identify these non-canonical antigens and develop programs that consider these modifications.

The dependence on using binding affinity as a major predictor of putative neoepitopes, while useful in some cases, may be limiting our ability to accurately identify all neoepitopes. The criteria of what makes a good neoepitope have yet to be established, but with the identification of more neoepitopes and consideration of weak affinity and non-canonical neoepitopes, we can start to identify and set the criteria that can help more accurately predict neoepitopes. Only then will we further understand what makes a good neoepitope.

How should we immunize against neoepitopes?

Since neo-antigens are small pieces of peptides harboring tumor mutations, immunization with them usually needs strong immunostimulatory agents to produce efficient immune response. Peptides as vaccines may not be able to stimulate the immune system powerfully enough on their own. Therefore, it is usually required to use an adjuvant in combination in order to elicit an effective immune response [45]. The word adjuvant comes from the Latin word adjuvare that means to enhance or help. The role of adjuvant is to enhance the immune system against the antigen which is co-inoculated[46].

In order to activate naïve cytotoxic T cells and have a robust immune response, usually stimulation of T helper (Th) cells is needed[47]. Synthetic peptides designed to stimulate cell mediated immunity often do not have a proper Th-epitope and therefore they are not efficient by themselves[48]. Even with the proper T cytotoxic and Th cell epitopes, an adjuvant is still required in order to have an effective peptide based vaccine[49]. Based on our laboratory's unpublished data on BALB/c mice, using a potent immunostimulatory adjuvant and delivery system for neo-epitope immunization seems crucial to have an effective tumor rejection response.

In order to get a maximized cytotoxic T lymphocyte response in cancer immunotherapy, besides proper neoepitope, appropriate adjuvant that can induce production of cytokines and co-stimulatory molecules from APCs is required. It is also important that the adjuvant delivers optimum amount of the antigen by controlling antigen persistence and concentration[50]. There should be a balance between antigen persistence and delivery. Long term antigen persistence at the site of injection may have adverse results in tumor rejection and cause CD8 T cell dysfunction and deletion[51]. Adjuvants function in different ways including releasing the antigen gradually[52,53], stimulating pattern recognition receptors on APCs[54,55], and protection of antigens from rapid degradation and therefore extending the antigen presentation time[56,57].

Different types of adjuvants have been tested in cancer therapy vaccines such as mineral adjuvants and cytokines[58], RNA based adjuvants[59], liposomes[60], tensoactive agents[61], and bacterial products[62]. In some studies, different types of cells were pulsed with neoepitopes for immunization. These cells including B cells[63], macrophages[64], splenocytes[38] and dendritic cells[65] serve as both delivery system and adjuvant.

Dendritic cells (DC) as professional antigen presenting cells play a major role in initiating immune response[66]. Since the early clinical trials testing effectiveness of DC vaccines in cancer,[63,67] it wasn't until 2010 that the first cancer antigen presenting cells/DC-based vaccine (Sipuleucel-T) got FDA approval[68]. Since DCs are able to efficiently uptake, process and present the antigen and initiate the immune response, they may be considered as natural adjuvants[69,70]. Although there are many studies using dendritic cells as delivery system for different cancer immunotherapies that are showing promising results on T cytotoxic cell response,[71–73] most of them failed to induce good tumor rejection[74,75,76,77]. One of the challenges in using DCs as delivery system in neoepitope cancer therapy is providing enough functional DCs for each patient that may not be achieved by isolating patient blood DCs[78]. The number of blood DCs in cancer patients often is reduced[79] and they may be functionally impaired due to tumor microenvironment systemic effect[80]. There are other ways to get syngeneic DCs including in-vitro differentiation of blood monocytes[81], hematopoietic stem cells[82] or bone marrow derived stem cells[83], in which each have their own hurdles. Different variables using DCs play a major role in the final anti-tumor immunity caused by dendritic cell neoepitope based cancer vaccine; such as DC maturation status[84,72], DC type, number of the injected DCs and injection location[85]. It is clear that having the appropriate tumor neoepitope is one of the most important elements in cancer immunotherapy by DCs. However, determining the exact DC subtype that works the best as adjuvant for immunotherapy may be one of the future directions in DC based vaccines.

Combinatorial Therapies

It is now beyond doubt that the immune system, and more specifically, the T cell compartment have the ability to control and eliminate tumor lesions. Neoepitopes sit at the forefront of cancer immunotherapy, in that they are the prospective means by which the immune system can distinguish normal self from mutated self and selectively eliminate only cancerous cells[86,87]. Further, many immunotherapies, traditional cancer treatment modalities (such as chemotherapy and radiation) and disease progression all potentially affect responses to neoepitopes either directly or indirectly[88,89]. However, even armed with the knowledge of neoepitopes and improved techniques and tools for identifying and targeting them *in vivo*, no "silver bullet" treatment has emerged for treating all cancers.

As discussed in the preceding sections, myriad advances have been made in the last few years with regards to the identification, importance and utility of neoepitopes. However, there remain significant hurdles in leveraging this information into effective treatment modalities. A particular challenge is with the treatment of solid tumors. While other related immunotherapies, such as chimeric antigen receptor (CAR) T cells, have shown considerable improvements in outcomes for many liquid or blood tumors, solid tumors have been more difficult to target with this approach[90] given that most solid tumors lack the well characterized surface markers found on blood cell subtypes. Further, factors within the tumor microenvironment (TME) can work in concert to create an immunosuppressive milieu that can limit the local effectiveness of a strong, systemic anti-tumor immune response[91]. TME elements such as suppressor cell types (Regulatory T Cells, Myeloid Derived Suppressor Cells, etc.), suppressive cytokines, extracellular matrix and vasculature remodeling, T cell exclusion, and others are all means by

which cancer cells can alter their environment and coopt the host immune system for their protection.

Specific therapies, such as immune checkpoint blockade, have been developed to target immunosuppressive components. The idea behind these treatments is that by targeting the co-inhibitory molecules CTLA-4 and PD-1 found on T cells, the inhibitory signaling or "brakes" can be removed from these cells thus generating a stronger anti-tumor response[92,93]. These therapies have generated exciting and durable responses for many different types of cancer[94–98]. However, these responses remain limited to only a subset of patients, which can differ significantly between tumor types. Determining biomarkers that can identify which subset of patients respond to treatment has become critical for both improving treatment outcomes and understanding their actions mechanistically. To this end, studies examining anti-CLTA-4 antibody therapy have demonstrated that its efficacy may be a result of depleting Regulatory T cells from the TME and not simply taking the "brakes" off of effector T cells[99–101]. The lack of a universally effective treatment method owes in part to the complexity of the tumor and its microenvironment. This complexity has been demonstrated not only in the significant disparities between different tumor types but further as the vast heterogeneity within a single patient's lesion(s)[89]. Numerous factors such as differences in tumor cell clonality, immune cell infiltration, angiogenic factors, mutational burden, etc. contribute to treatment response and ultimately determine treatment outcomes.

It is becoming increasingly evident that while individual immunotherapeutic regimes have exciting potential, it will likely take a combinatorial, and more importantly, patient specific

approach to achieve optimal treatment outcomes on a patient-by-patient basis[102]. To this end, trials examining the combination of checkpoint inhibition therapies using antibodies targeting CTLA-4 and PD-1/PD-L1 have shown synergistic improvements for some patients[103]. Yet, considerable side effects of such combinatorial treatments remain a challenge. The development of other therapies that target additional co-inhibitory or co-stimulatory molecules is also underway. Other T cell surface molecules such as the co-inhibitory markers LAG-3 and TIM-3 and the costimulatory receptors OX40, GITR, and 4-1BB are currently under evaluation individually and in combination with other checkpoint based therapies for their utility as anti-cancer agents[104]. Given the specific TME milieu for each individual tumor, precise checkpoint targeting may improve outcomes for certain subsets of patients.

Even as other immunotherapies become more "patient specific", neoepitopes endure as the most targeted approach to cancer treatment. The identification of patient specific neoantigens allows for a directed immune response to tumor specific epitopes. There are many different approaches examining how best to elicit a strong, specific and long-lasting neoepitope immune response. One set of methodologies revolves around a vaccine-based approach. As previously described in this review, a host of different vaccination strategies from synthesized peptides to whole cell or cell lysate products in a mixture with adjuvant can be used as cancer targeted vaccines. Other *ex vivo* based methods such adoptive cell transfer (ACT) or CAR–T cell aim to selectively expand or engineer T cells with specificity to neoepitopes and re-infuse these cell products back into the patient. The overarching goal of neoepitope therapies is to effectively "educate" the immune system to the best tumor specific targets while eliciting a strong T cell based response that can hopefully overwhelmed a tumor's defense mechanisms and elicit complete tumor rejection[12].

The future of neoepitope based cancer immunotherapies

It is evident that all the components of a neoepitope-based therapy, from their *in silico* identification to optimal immune system activation, are critical to realizing their therapeutic potential. While neoepitope targeted approaches have moved from the laboratory to the clinical setting, outcomes have remained varied and suboptimal. Much in the same way as other immunotherapies demonstrate durable responses for only a subset of patients, utility of neoepitope focused strategies are likely dependent on the context of the tumor and the TME. As more information continues to clarify our understanding of the interrelated cancer immunology mechanisms such as TME associated suppression, T cell exclusion, T cell exhaustion, immune checkpoint regulation, tumor heterogeneity, etc. it is becoming clear that no single targeted approach will be sufficient to treat the vast majority of cancers. Moving forward, optimal therapeutic advances will likely need to include multiple immune directed treatments that are tailored not only to the patient's specific neoepitope repertoire but also to the unique suppressive landscape of the individual TME.

For my thesis, I set aside all preconceived notions of what makes a good neoepitope. Instead of predicting what neoepitopes to use in a vaccine, I tested every somatic mutation's epitope for its ability protect a host from tumor challenge. In doing so, I identified the best candidate neoepitopes for a given murine tumor cell line, and have begun to understand the criteria that make for a strong candidate neoepitope. Through this exhaustive and exhausting study I have made substantial discoveries, and identified many more scientific questions that need to be

investigated further before neoepitopes become a major contender in personalized cancer immunotherapies.

Chapter 2

Materials and Methods

Location of research facilities

All experimental procedures on live animals were performed in B1005A (Procedure Room) in the animal facility at UConn Health, Farmington. All experiments not involving live animals were performed in Dr. Pramod K. Srivastava's laboratory (Room L1008). Accordingly, all the apparatus and reagents required for the experiments were provided by Dr. Srivastava. Flow cytometry was performed either in Dr. Srivastava's laboratory or at the flow cytometry core facility located in room E6014 at UConn Health. FlowJo, a software to analyze the data recorded using flow cytometers, was made available by Dr. Srivastava's laboratory. Experiments measuring the binding affinities of synthetic peptides to MHC class I molecules were performed by Dr. John Sidney in the laboratory of Dr. Alessandro Sette at La Jolla Institute for Allergy and Immunology in San Diego, CA. Experiments using molecular modeling were performed by Dr. Brian Baker at the University of Notre Dame in Notre Dame, IN.

Vertebrate Animals

In this study, 6-8 week old female C57BL/6 mice from Jackson Laboratories™ were used. Mice were housed and cared for by the Center for Comparative Medicine at the University of Connecticut Health, and all procedures can be found in the approved Srivastava protocols, reference number 101350-0619.

MC38-FABF cell line

A chemically induced murine tumor cell line in the C57BL/6J background known as MC38-FABF was used as the primary tumor model for this extensive study. MC38-FABF tumor cell line was graciously provided by Dr. Alan B. Frey at NYU Langone Health.

Sequencing

Sequencing of cDNA was performed by the Illumina NextSeq 500 Sequencing System. Sequencing was performed following the EpiSeq pipeline as previously described by Duan et al[38]. SNVs were called using normal mouse reference sequences. Whole exome sequencing was followed by transcriptome sequencing to call expressed SNVs with the least incidences of false positives. Further, to validate these SNVs, Sanger sequencing was performed for all SNVs.

Culture Complete Media

All cells were cultured in RPMI that are supplemented with 10% FBS, 1% penicillinstreptomycin-glutamine, 1% MEM, lx beta-mercaptoethanol, and 1% sodium pyruvate.

Generation of BMDCs

BMDCs were generated as per Inaba et al[105]. Bone marrow from femurs and tibias of ~6-8 week old mice were cultured with 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) in complete RPMI media for 7 days at 37˚C and 5% CO_2.

Peptides

The peptides used in this thesis were ordered from JPT in Berlin, Germany. They were synthesized with a purity of >90%. The peptides were dissolved in DMSO at a final concentration of 20 mM and stored at -20˚C.

Immunizations

BMDCs, ~7x10^6 cells, were pulsed with 100µM of peptide in RPMI complete media for ~1 hour at 37˚C and 5% CO_2.. They were washed once in RPMI only. Mice were immunized twice, a week apart with ~6 million pulsed BMDCs intradermal in the belly.

Tumor challenge and representation of tumor growth.

Mice were challenged with 30,000 tumor cells intradermally in the right rear flank. Tumor volumes were measured every 3 days post tumor challenge using the Biopticon TumorImager™. Mice were euthanized when tumors reached a volume of 2000mm^3. Tumor Control Index scores were calculated for every experiment as described by Corwin et al[106].

Tumor sample preparation

Tumors were harvested on day 25 post tumor challenge. Tumors were dissociated using Miltenyi™ Tumor Dissociation Kit, mouse. $CD8^+$ TIL were isolated with STEMCELL's EasySep™ murine CD8 negative selection kit.

Intracellular IFNγ assay by ELISpot.

$CD8^+$ cells were isolated from spleens of mice immunized with neoepitopes using the magnetic bead isolation (Miltenyi Biotec™). Negative controls were $CD8^+$ cells from immunized mice without peptide stimulation. IFNγ ELISpot reagents were obtained from MABTECH™. As targets to stimulate the $CD8^+$ cells, naive splenocytes pulsed with peptide were added to the wells. Plates were analyzed by ZellNet™. Peptides were considered to have a positive CD8

response when spots from peptide-stimulated wells were significantly higher than that without cognate peptide stimulation.

Depletion of T cell subsets.

$CD8^+$ cells were depleted in immunized mice using anti-CD8 rat IgG2b monoclonal antibody 2.43 (Bioxcell™). CD4 cells were depleted using anti-CD4 rat IgG2b monoclonal antibody GK1.5 (Bioxcell™). Isotype control groups were given anti-LTF2 rat IgG2b monoclonal antibody (Bioxcell™). Depleting antibodies were given in PBS i.p. 2 days before each immunization and every 7 days for the duration of the experiment. The first 2 injections of depleting antibodies given before each immunization were 250 µg per mouse; later injections were 100 µg per mouse. The antagonistic antibody, anti-CTLA-4 (clone 9D9; Bioxcell) was given at 75 µg, 7 days before and every 3 d after tumor challenge. The appropriate T cell subsets were depleted by >95%.

Flow Cytometry

The plasticity flow panel was graciously provided by Dr. Andrea Schietinger at MSKCC. The antibodies for CD8α PacificBlue (clone 53-6.7), CD38 APC (clone 90), Lag3 (clone eBioC9B7W), PD-1 PCP-Cy5.5 (Clone RMP1-30), Tim3 APC (Clone RMT3-23), and CD62L APC-Cy7 (Clone MEL-14) were purchased from Biolegend™. The antibody for 2B4 PE-Cy7 (Clone eBio244F4) was purchased from ThermoFisher™. The antibody for TCF1 Alexa Flour 488 (Clone C63D9) was purchased from Cell Signaling™. Ab Flow cytometry was performed using Miltenyi Biotec MACSQuant analyzer. Analysis was done using FlowJo software (FlowJo LLC).

Molecular Modeling

Modeling was performed by Grant Keller in the lab of Dr. Brian Baker at the University of Notre Dame. Molecular modeling was performed for the precise neoepitopes that showed a significant tumor response. Low-resolution centroid modeling was followed by high-resolution refinement and then repeated 10,000 times to find the highest likely conformation of the neoepitope and its wild type counterpart. PCA analysis was conducted on density-based spatial clustering of applications with noise (DBSCAN) to find the modeled populations with the favorable lowest energetic scores. Differences were also quantified by superimposing average peptide conformations from the molecular dynamics simulations and computing RMSDs for all common atoms. Cox6a2 was modeled for H-2K^{b} and Fam171b was modeled for H-2D^{b}.

Statistical analysis

P-values for group comparisons were calculated using a two-tailed nonparametric Mann–Whitney test, using GraphPad Prism 5.0 (GraphPad). Fisher's exact test was used to test association between pairs of categorical parameters. Statistical significance of a Pearson correlation coefficient was computed using two-sided Student's t test as described in Cohen et al[107]. Statistical analysis on percent survival curves was conducted using the log rank (Mantel-Coz) test.

Chapter 3

Identification and characterization of tumor protective neoepitopes

Introduction

It is evident that using the binding affinity of a neoepitope to its cognate MHC may be a limiting factor in designing a tumor rejecting vaccine. With less than 1% of predicted high affinity antigens eliciting a tumor specific immune response, the odds of accurately selecting the true and effective neoepitopes for vaccination are very low[42]. It is even more difficult to accurately predict multiple neoepitopes that can account for the heterogeneity of a tumor that may only contain one of the targets at a time. Evidence of this can be seen in patients treated with various cancer immunotherapies and the tumors escape with neoantigen loss[108–110]. Until this study, all neoepitopes in a murine model system have been identified using binding affinity as a criterion and only identified a few neoepitopes, as shown in Table 2. The study, by Duan et al, was able to predict the most neoepitopes for a tumor cell line that were capable of rejecting tumor growth[38]. Using DAI as a prediction criterion may be the most accurate and is also consistent with a large amount of clinical data in patients that received checkpoint blockade[111,112]; however this algorithm still needs to be improved upon.

According to the NIH U.S. National Library of Medicine Clinical Trials there are more than 80 clinical studies either testing or looking at the neoepitopes in various cancer types[113]. As more data evolves, we will further begin to understand the efficacy of various neoantigen selection strategies. Some early studies solely identified the presence of neoepitope specific $CD8^+$ and $CD4^+$ TILs of melanoma patients that had responded to checkpoint blockade[114–118]. The first completed human vaccine Phase I trials in advanced melanoma patients tested the safety and efficacy of neoepitope vaccines using neoantigen pulsed DCs, synthetic long peptide (SLP) and polyepitope mRNA neoantigen vaccines.

Table 2 | Genomically defined murine neoepitope studies

Paper	No. of models tested for tumor rejection	No. of neoepitopes tested for tumor rejection	No. of neoepitopes found positive for tumor rejection
Brennick, George, Srivastava (Unpublished)	**1 (MC38-FABF)**	**279 (within groups)** **120 (as individuals)**	**9**
Ebrahimi-Nik, Srivastava (Unpublished)	**1 (MethA)**	**Long peptide- 7** **Precise peptide- 11**	**Long peptide- 4** **Precise peptide- 5**
Yadav, Nature, 2014[40]	**2 (MC-38, TRAMP-C1)**	**MC-38- 3** **TRAMP-C1- 0**	**MC-38- 3** **TRAMP-C1- 0**
Gubin, Nature, 2014[39]	**2 (d42m1-T3, F244)**	**d42m1-T3- 2**	**d42m1-T3- 2**

This table lists the important genetically identified neoepitope papers that have set the foundation for all human clinical neoepitope studies, and lists how many neoepitopes were tested and successful at rejecting tumors.

Duan, J.Ex. Med, 2014[38]	**2 (MethA, CMS5)**	**<u>High affinity</u> MethA-11 CMS5- 7 <u>DAI</u> MethA- 28 CMS5- 20**	**<u>High affinity</u> MethA-0 CMS5- 0 <u>DAI</u> MethA- 4 CMS5- 6**
Castle, Cancer research, 2012[119]	**1 (B16F10 melanoma)**	**2**	**2**
Kreiter, Nature, 2015[120]	**3 (B16F10, CT26, 4T1)**	**B16- 1 CT26-10**	**B16-1 CT-26- 5**

This table lists the important neoepitope papers that have set the foundation for all human clinical neoepitope studies, and list how many neoepitopes were tested and successful at rejecting tumors.

The first trial by Carreno et al, looked for neoepitope specific T cell responses in patients immunized with DCs pulsed with seven of the highest predicted binding affinities to the patients HLAs[121]. They demonstrated that they were able to successfully expand pre-existing neoepitope specific T cells and identify new TCR clones not seen before treatment. However, this study did not report the tumor responses of these patients. The other two studies by Ott et al and Sahin et al that used similar binding affinity prediction algorithms, did report the patient outcomes[122,123]. In the study by Ott et al, they vaccinated patients with SLPs post-surgical resection and looked primarily at T cell responses to the immunized peptides. Although the peptides were selected for electing $CD8^+$ responses, the overwhelming responses observed were $CD4^+$ T cells. Even though clinical observations were reported, the number of patients tested in both studies (six in Ott et al and thirteen in Sahin et al) was too small to obtain any information about vaccine dependent clinical activity. Interestingly, roughly 20% of the measurable T cell responses by Sahin et al were predicted to have a poor binding affinity to HLA class I and II. By solely using binding affinity criteria alone, these neoantigens would have been overlooked in any other study. However, the measured binding affinities of these neoantigens were not measured, therefore it is not known if they are truly weak binders or the predicting algorithms are not accurate.

In this thesis, I did not use binding affinity as a criterion to identify putative neoepitopes. I have used exome and RNA sequencing to identify all expressed somatic mutations, design 21mer SLPs, and tested them for tumor rejection capabilities in a C56BL/6 model. This allowed us to identify all neoepitopes for a single cancer cell line, and I was then able to begin to dissect what makes them strong neoepitopes. Since previous studies have debated the importance of $CD4^+$ and $CD8^+$ T cells in tumor rejection and specifically orchestrating bystander killing[124], I

determined the specificity of the neoepitopes. Furthermore, I tested the necessity of having multiple neoepitope targets to overcome antigen escape.

This study will not only identify neoantigens, but will aim to shed light on what factors contribute a neoepitope specific tumor rejection. It will generate vast amounts of data that can be used later on to extensively identify the criteria that are necessary for selecting the strongest candidate neoepitopes. It is only once we truly understand what makes a good neoepitope that personalized neoepitope vaccines will be an effective treatment modality.

Results

Identification of SNVs in MC38-FABF tumor cell line

A chemically induced murine colon cancer cell line in the C57BL/6J background, known as MC38-MC38-FABF, was used as the primary tumor model for this extensive study. This cell line develops an aggressive tumor and does not response to checkpoint blockade on its own. In order to identify all possible SNVs expressed by MC38-FABF, the EpiSeq pipeline as previously described by Duan et al[38] was used. SNVs were called using normal mouse reference sequences. Whole exome sequencing was followed by transcriptome sequencing to call expressed SNVs with the least incidences of false positives. Exome sequencing revealed 1883 SNVs and transcriptome sequencing brought the number of expressed SNVs down to 328. Further, to validate these SNVs, sanger sequencing was performed for all SNVs called. This again reduced the number of expressed SNVs to 279 (Figure 2). For these candidate neoepitopes, 21 amino acid long peptides were generated, with the mutation at the center of the peptide. This is to take into consideration all putative 8-11mers that can be presented by the major histocompatibility complex (MHC) class I.

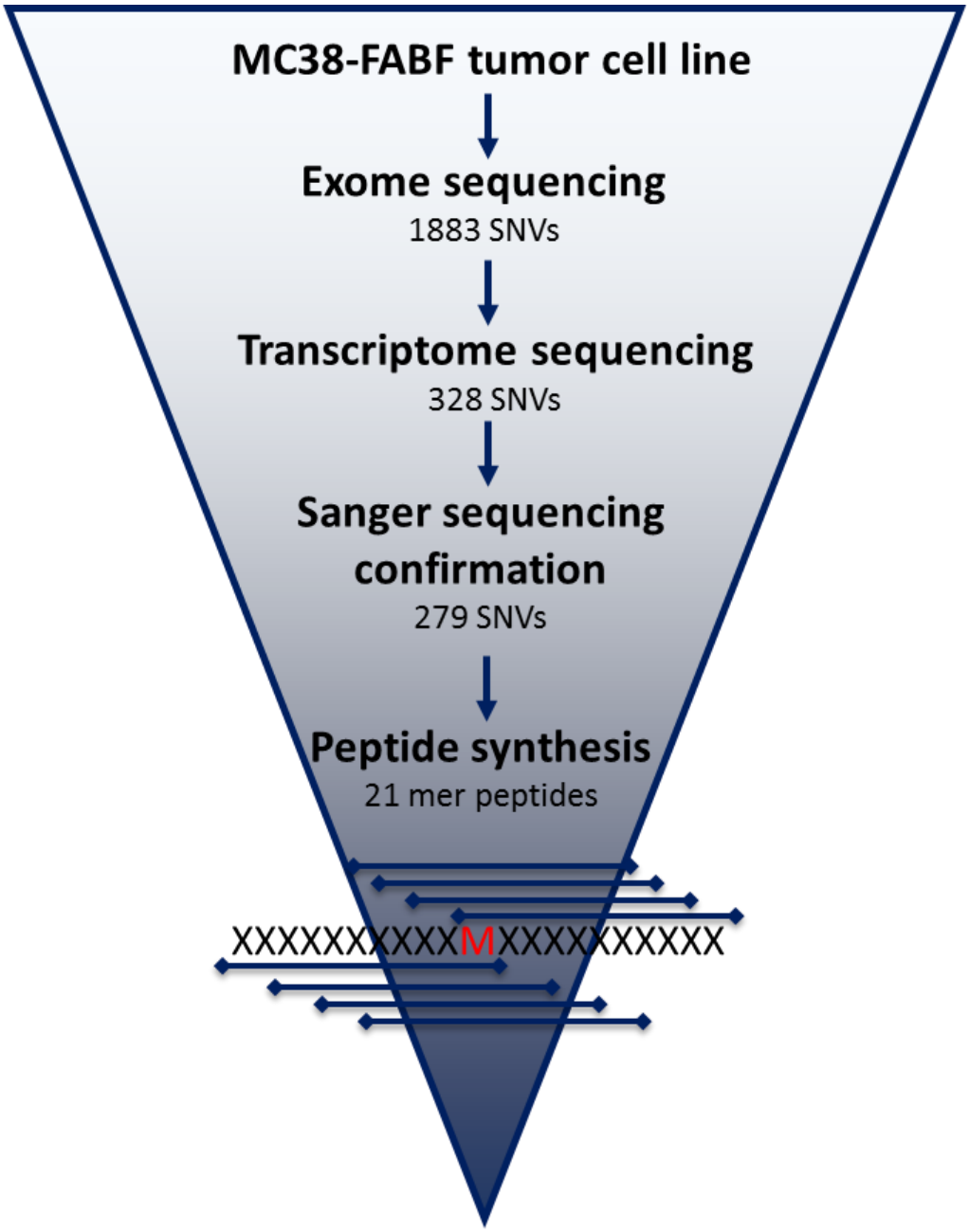


Figure 2 | Pipeline for epitope calling
Represented is a broad overview of the sequencing pipeline for calling somatic mutations of the MC38-FABF cell line.

Predicted neoepitopes are capable of eliciting tumor protection

MC38-FABF is capable of inducing progressively growing tumors, which is lethal in an untreated syngeneic host. Furthermore, MC38-FABF is immunogenic as it is capable of eliciting protection against subsequent tumor challenge upon immunization with irradiated tumor cells prior to challenge (Figures 3a-c). In order to test that the tumor protection is neoepitope specific, mice were immunized with a cocktail of all 279 synthetic peptides (Figure 3d). This strategy resulted in reduced tumor growth, but did not result in complete protection like the irradiated tumor when tested as a vaccine. The irradiated cells themselves may contain other factors that are responsible for the complete protection, which needs to be investigated further. Since the 279 peptide vaccine showed significant tumor control over the BMDCs alone group, I next wanted to further identify the SNVs that are responsible for this observed response.

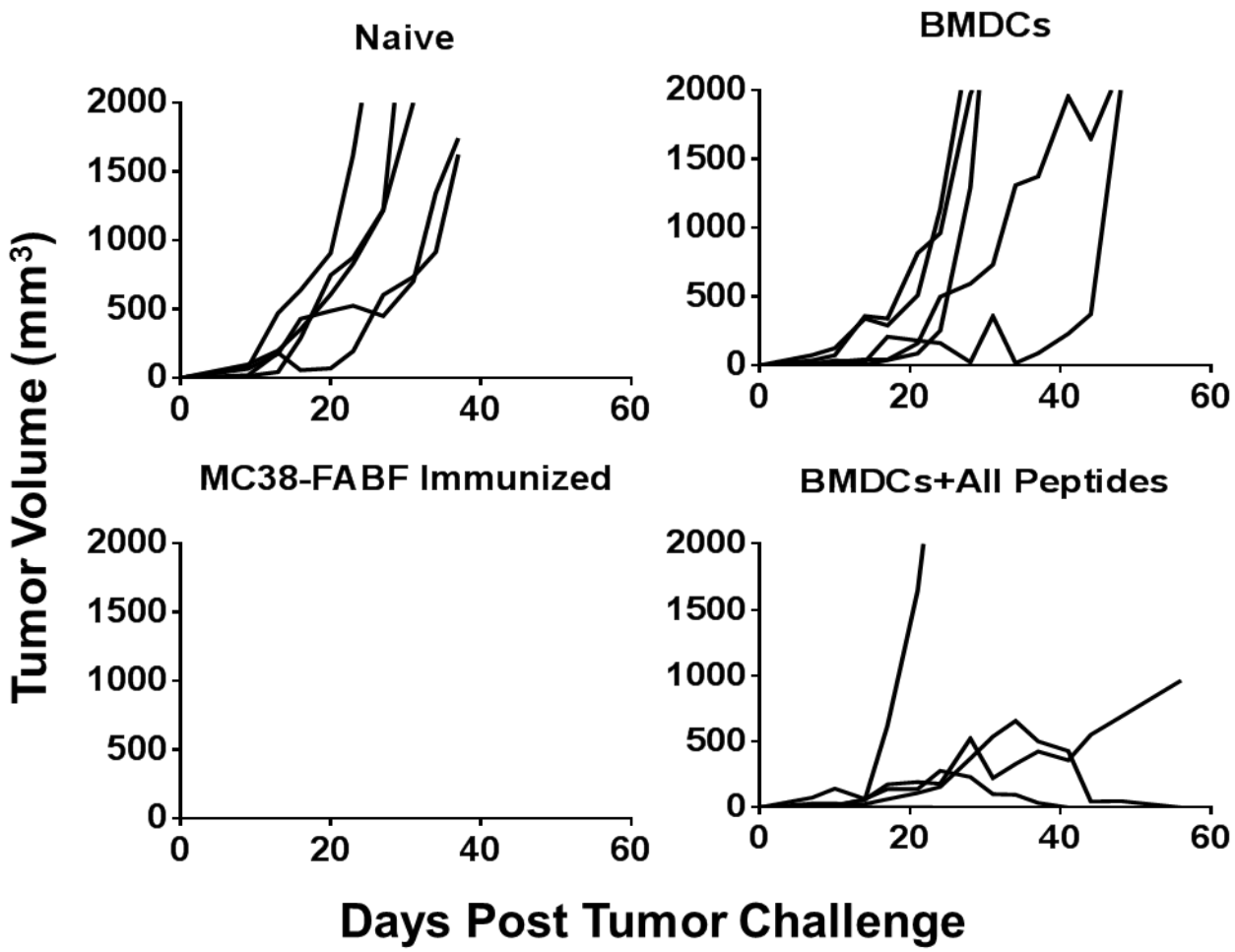


Figure 3| MC38-FABF immunizes against itself and has tumor protective epitopes
a-b. MC38-FABF intradermal injections induce tumors in C57BL/6 mice that have not received any vaccination (naïve) or BMDC control. **b.** Mice immunized with irradiated MC38-FABF cells elicit complete protection from tumor challenge. **c.** Mice were vaccinated with BMDCs pulsed with all 279 peptides and tumor growth curves are shown; **(a-c)** n=5 mice/group

Screening of all potential neoepitopes

The 279 peptides were randomly grouped into pools of five peptides per group and tested for tumor rejection following prophylactic immunization. Since these were long peptides that required processing for MHC class I presentation, bone marrow derived dendritic cells (BMDCs) were used as a neoepitope delivery system. Studies from our lab have shown that BMDCs outperform other neoepitope delivery systems[125]. Further, to prevent discrepancies in peptide uptake due to competition, BMDCs were separately pulsed with each individual neoepitope from the group. The individually pulsed BMDCs were then pooled together and used to immunize mice via the intradermal route followed by tumor challenge with live tumor cells. The immunization protocol has been previously described and illustrated along with the screening plan in Figure 4. Tumor control index (TCI) scores as described by Corwin et al[106], were calculated for all 56 groups tested and normalized to the control group (BMDC only) as shown in Figure 5.

Of the groups tested, just over 42% (24/56 groups) had a positive score, with only two groups showing significant tumor control (Figure 5a). The 120 peptides within these groups were selected for further testing for their capacity to elicit a tumor response as an individual peptide immunization. TCI scores for the individual neoepitopes were calculated as shown in Figure 5b. Of the 120 peptides tested, 50% (60 peptides) showed a positive TCI score. Only 7.5% (9 peptides) of all the individual peptides tested showed significant tumor control when compared the control groups.

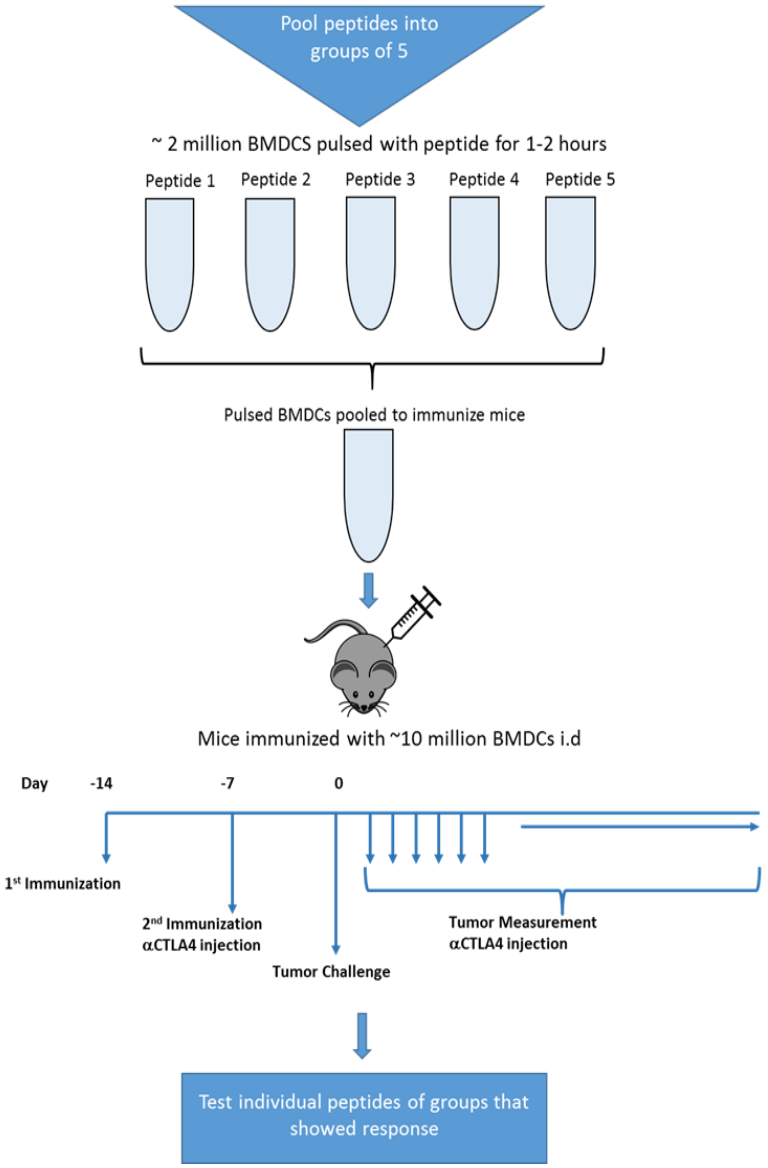


Figure 4 | Peptide screening strategy
Represented is the experimental scheme for rapidly and efficiently screening all 279 peptides for their ability to elicit tumor rejection.

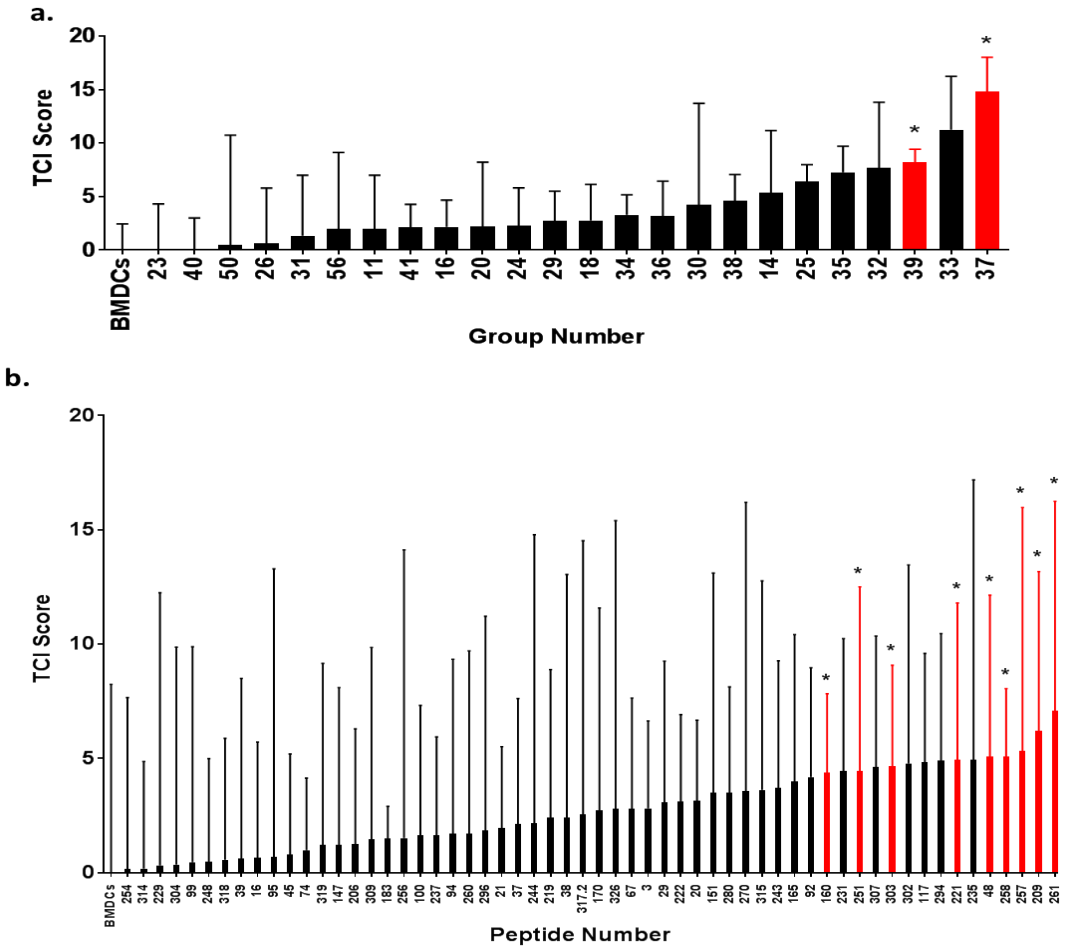


Figure 5| Positive tumor control index (TCI) scores of grouped peptides and the individual peptides of those groups
These figures represent data from peptides which eleicited a positive tumor control index (TCI) scores. **a.** The x-axis corresponds to the group number label of 5 peptides tested. TCI scores were calculated for each group, and compared to the BMDC control group for significance; n=5 mice/group. **b.** Individual peptides tested from groups with a positive TCI score. The x-axis corresponds to the peptide number tested. TCI scores were calculated for each group, and compared to the BMDC control group for significance as described by Corwin et al[98]; n=10 mice/group **(a-b)** Means ± s.d. shown.; red *p<0.05 (Student's t- test).

Multiple neoepitopes shows additive effect in tumor control

I made the observation that the top performing grouped vaccines in the initial screening (Figure 5a) did not result in individual neoepitopes that were able to elicit significant tumor control. For example, the top performing group 37 showed significant tumor control as a group, but when tested as individual neoepitope vaccines did not show any significant control. Interestingly, when taking the sum of the TCI scores for the individual peptide vaccines, they almost exactly add up to the group score. This may suggest that neoepitopes are additive in their effectiveness, and multiple targets may be necessary for an effective treatment modality.

a.

Immunization	TCI Score
229	0.3
231	4.4
235	4.9
237	1.7
243	3.7
Total	**15.0**
Group 37	**14.8**

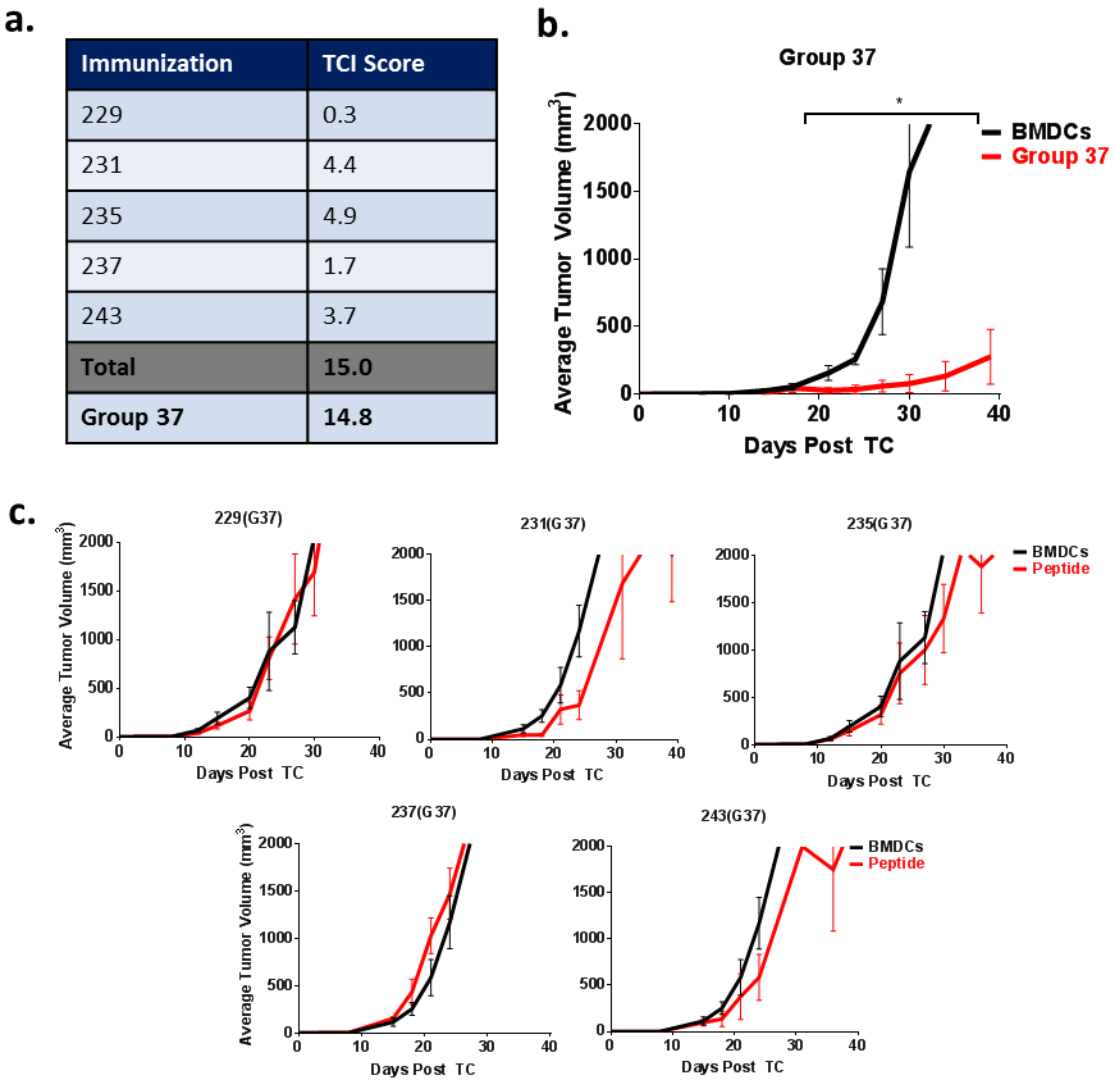


Figure 6 | Multiple targets show additive effect in tumor control
a. The table contains TCI scores for each of the individual peptides tested in Group 37. The sum of the scores are nearly identical to the TCI score of all the peptides as a grouped vaccine. **b.** The average tumor growth curves for Group 37 vaccination; *p<0.05 (Student's t-test) **c.** The average tumor growth curves for every individual peptide in Group 37. Means ± s.e.m. shown. **(a.b.)** n=5 mice/group

In order to test this hypothesis, I combined all 279 peptides into a single vaccine and only the top nine significant neoepitopes into another vaccine and tested the tumor growth responses. The combination of the top nine neoepitopes outperformed all vaccines tested to date (Figures 7a-b), confirming that multiple targets have a stronger effect than a single neoepitope. In addition, having selected only identified effective and relevant neoepitopes rather than all SNVs, regardless of performance, increased the vaccine efficacy.

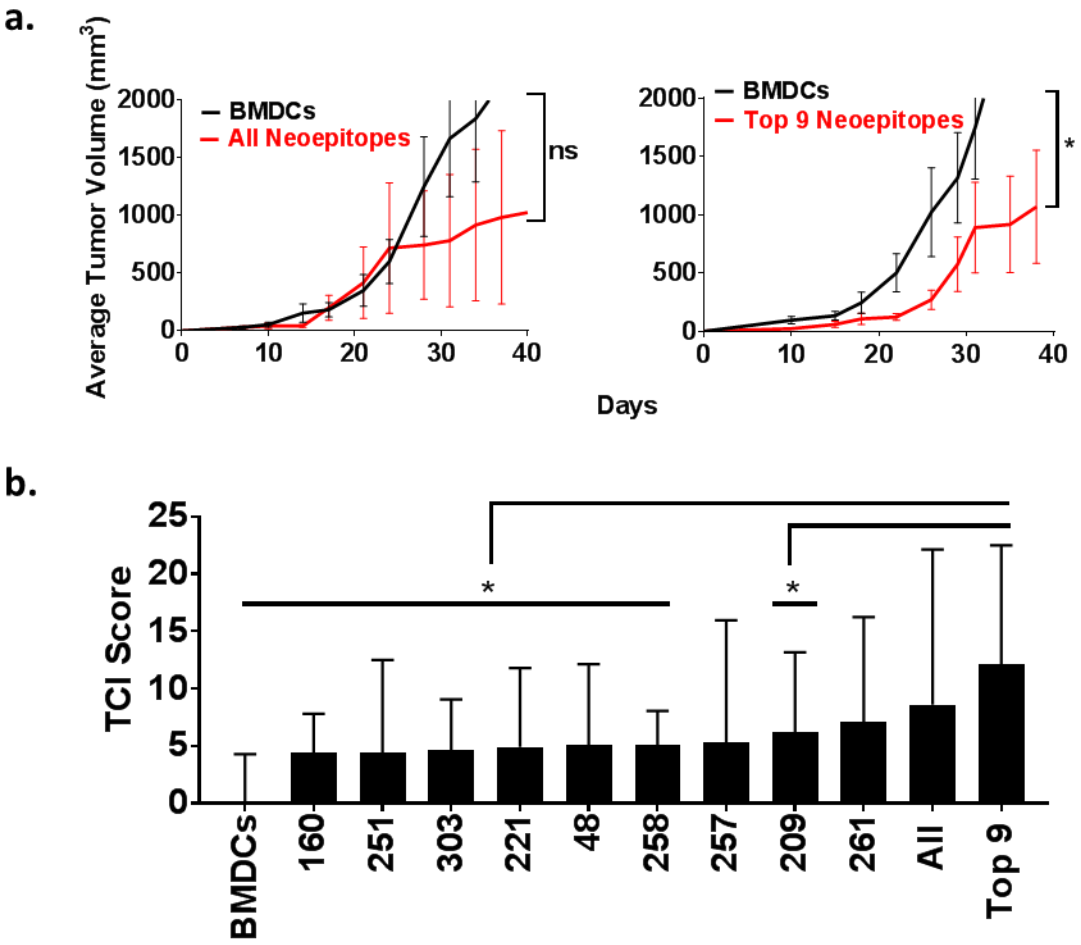


Figure 7 | Combining neoepitopes elicit the strongest tumor response
a. Combining all 279 peptides into one vaccine show slowed tumor growth, although not to a statistically significant degree. Combining only the top 9 neoepitopes showed significantly slowed tumor growth over the controls, and is the best performing vaccine in this study to date. Means ± s.e.m. shown. **b.** TCI scores are represented for the top 9 peptides as individual vaccines, all 279 peptides in one, or the top 9 in one vaccine. Means ± s.d. shown. **(a.b.)** n= 10; $p<0.05$ (Student's t-test).

Detectable tumor specific $CD8^+$ T cell response does not always result in tumor protection

Since we know that MC38-FABF immunized mice elicit complete protection to subsequent tumor challenge, I wanted to test which neoepitopes are recognized by these mice. Mice were immunized twice with irradiated MC38-FABF tumor cells, and then $CD8^+$ enriched cells from the spleens of these mice were tested for their recalled IFNγ response to all 279 peptides pulsed onto splenocytes via ELISpot. Of the 279 peptides tested only 30% (84/279) showed any positive T cell response with 9.4% (26 peptides) showing a significant response (Figure 8). Within the nine peptides that showed significant tumor response in the previous tumor rejection screening, only two peptides were observed to have a significant $CD8^+$ response against MC38-FABF immunized mice.

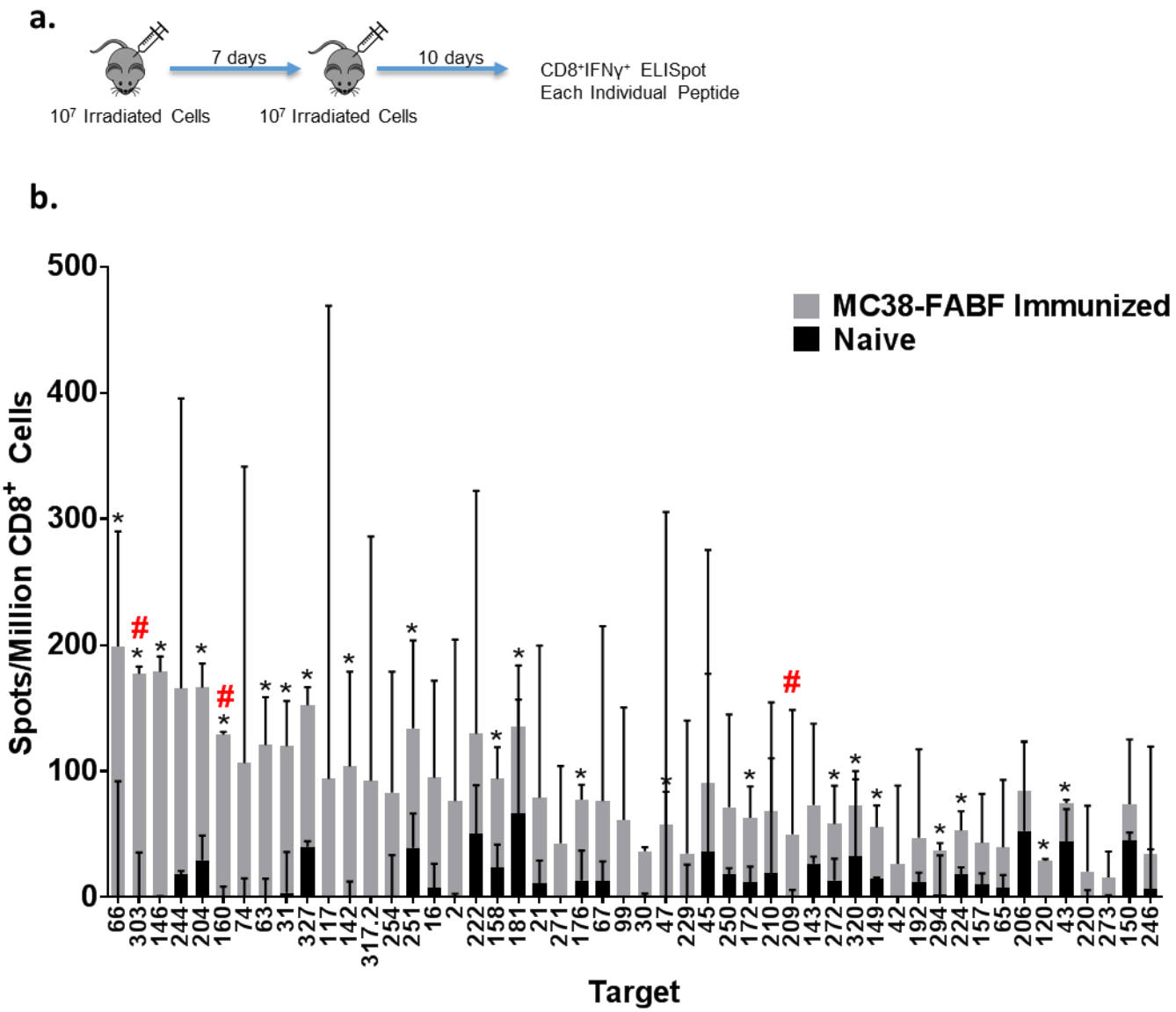


Figure 8 | Top 50 CD8+IFNγ+ responses against peptides of mice immunized with irradiated tumor MC38-FABF
a. Experimental scheme. **b.** This data shows to top 50 immunogenic peptides that recalled responses against MC38-FABF immunized mice. Splenocytes pulsed with each of the 279 peptides were used as targets. Each grey and overlaying black bar represents the response against MC38-FABF immunized mice and naïve mice respectively (n=4). Statistical analysis was conduct for each peptide response against wells with no target. Means ± s.d. shown; *p<0.05 (Student's t-test); #(red) peptide elicited significant tumor control.

Weak binding affinity neoepitopes outperform high affinity neoepitopes in tumor rejection

After extensive testing of all the individual peptides within groups with a positive TCI score, I found nine peptides that caused significant tumor control (Table 3). Only a single peptide was within the top 10% of predicted binding affinities for both H-2K^b and H-2D^b. Of the neoepitopes that had a significant tumor response, only two are predicted to bind MHC class I. Furthermore, the highest tumor control scoring neoepitopes have a predicted binding affinity well above the minimum 500 nM threshold[37]. This data suggests that binding affinity alone is not a predictor of a strong tumor response and through only screening putative neoepitopes below the 500 nM threshold, the best candidate neoepitopes for tumor rejection would be overlooked.

Table 3 | Top nine neoepitopes' predicted binding affinities

Predicted Binding Affinities						
Peptide #	**TCI Score**	**Gene Name**	**Kb IC50**	**Db IC50**	**Kb DAI**	**Db DAI**
261	7.08	Cox6a2	8071.7	31233.9	1.01	0.13
209	6.21	Fam171b	3083	26004	1.96	0.88
257	5.3	Psma1	8799.1	24703.7	0.97	0.41
258	5.08	Plk1	2759.4	18038.9	1.46	1.18
48	5.07	Kif3a	2050.1	25588.9	0.86	0.10
221	4.93	Oas3	27345.7	27856.1	0.34	0.01
303	4.66	Sh3rf1	331.6	3349.6	2.81	0.36
251	4.45	Tpra1	14.8	9326.3	1.02	0.74
160	4.38	Atg9a	9935.5	9463.1	0.47	2.19

Table shows specifics about each of the top nine working peptides. IC_{50} scores (nM) for H-2K^{b} and H-2D^{b} are predicted using the NetMHC4.0 algorithm. DAI scores are calculated as per Duan et al. Peptides highlighted in red elicited a significant IFNγ response in MC38-FABF immunized mice.

The strongest candidate neoepitopes Cox6a2 and Fam171b

The top two neoepitopes that have consistently showed the strongest activity in terms of tumor regression are namely 261 and 209, now referred to by their gene names, Cox6a2 and Fam171b respectively. (Figure 9a). In addition overall tumor growth control, the survival of mice immunized with either neoepitope was significantly longer compared to control mice (Figure 9b). Wild type counterparts of Cox6a2 and Fam171b do not elicit any tumor protective confirming the specificity of these neoepitopes (Figure 9c). In order to test previous observations that neoepitope tumor control is additive, I tested the combination of both Cox6a2 and Fam171b. In concurrence with earlier observations, the combination of Cox6a2 and Fam171b overall performed better than as individuals (Figures 10b-c).

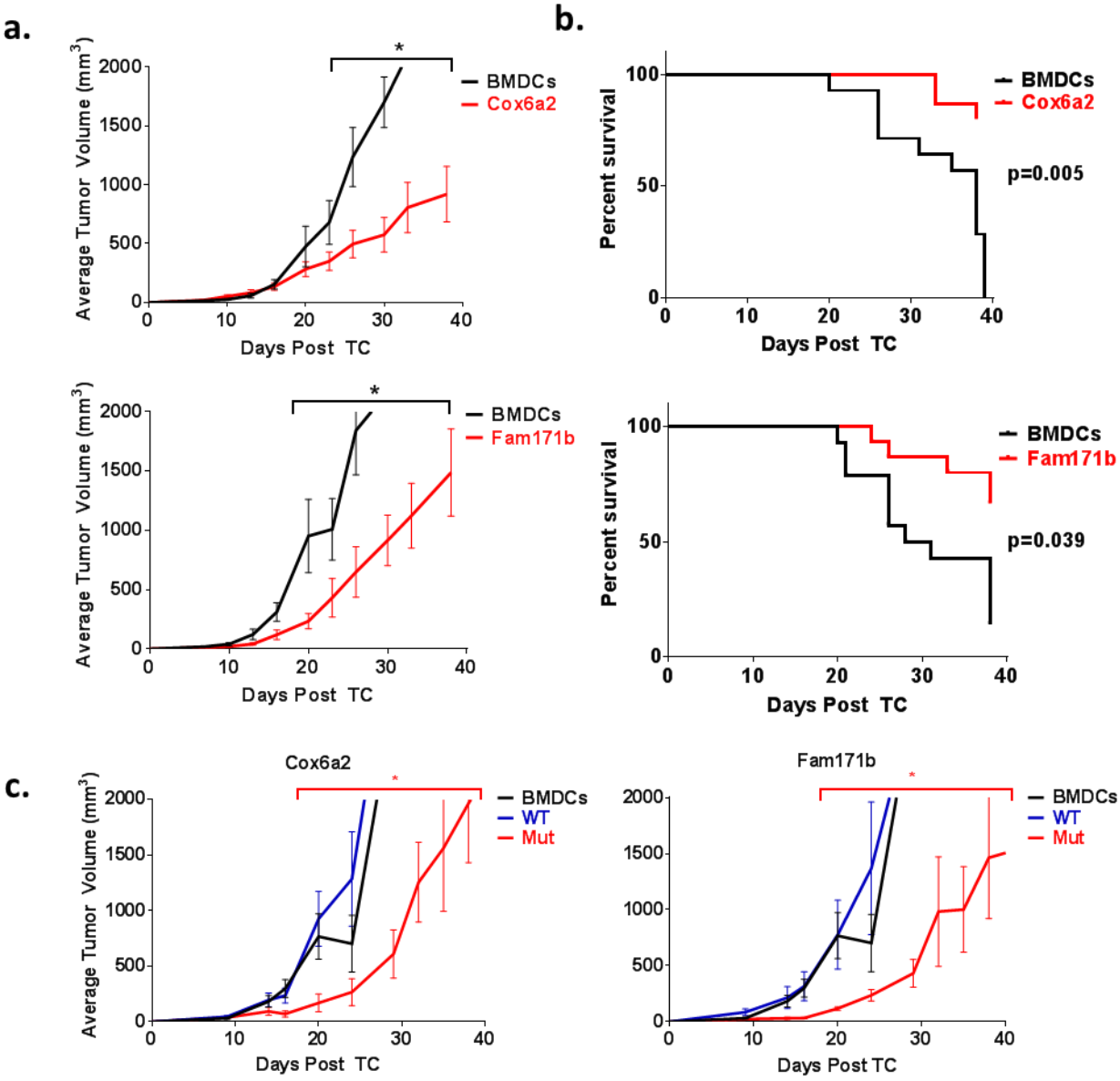


Figure 9| The strongest candidate neoepitopes Cox6a2 and Fam171b
a. The average tumor growth curves for BDMCs only compared to peptide 261 or 209. Means ± s.e.m. shown. **b.** Percent survival curves for BDMCs only compared to peptide 261 or 209. Statistical analysis was performed using the log rank (Mantel-Coz) test. **(a.b.)** n=15 mice/group **c.** The average tumor growth curves for BDMCs only compared to the mutant or wild type peptides of 261 or 209. Means ± s.e.m. shown; n=5 mice/group. **(a.c)** *p<0.05 (Student's t-test)

Until now, all neoepitopes were tested in a prophylactic model. I next wanted to test these neoepitopes in a therapeutic model, where vaccination does not occur until tumor challenge (Figure 10a). Both Cox6a2 and Fam171b showed significant tumor control in a therapeutic model, with Cox6a2 showing significant increase in survival. As observed previously, the neoepitopes performed best in combination for therapy, confirming that a combination of neoepitopes works best (Figures 10d-e).

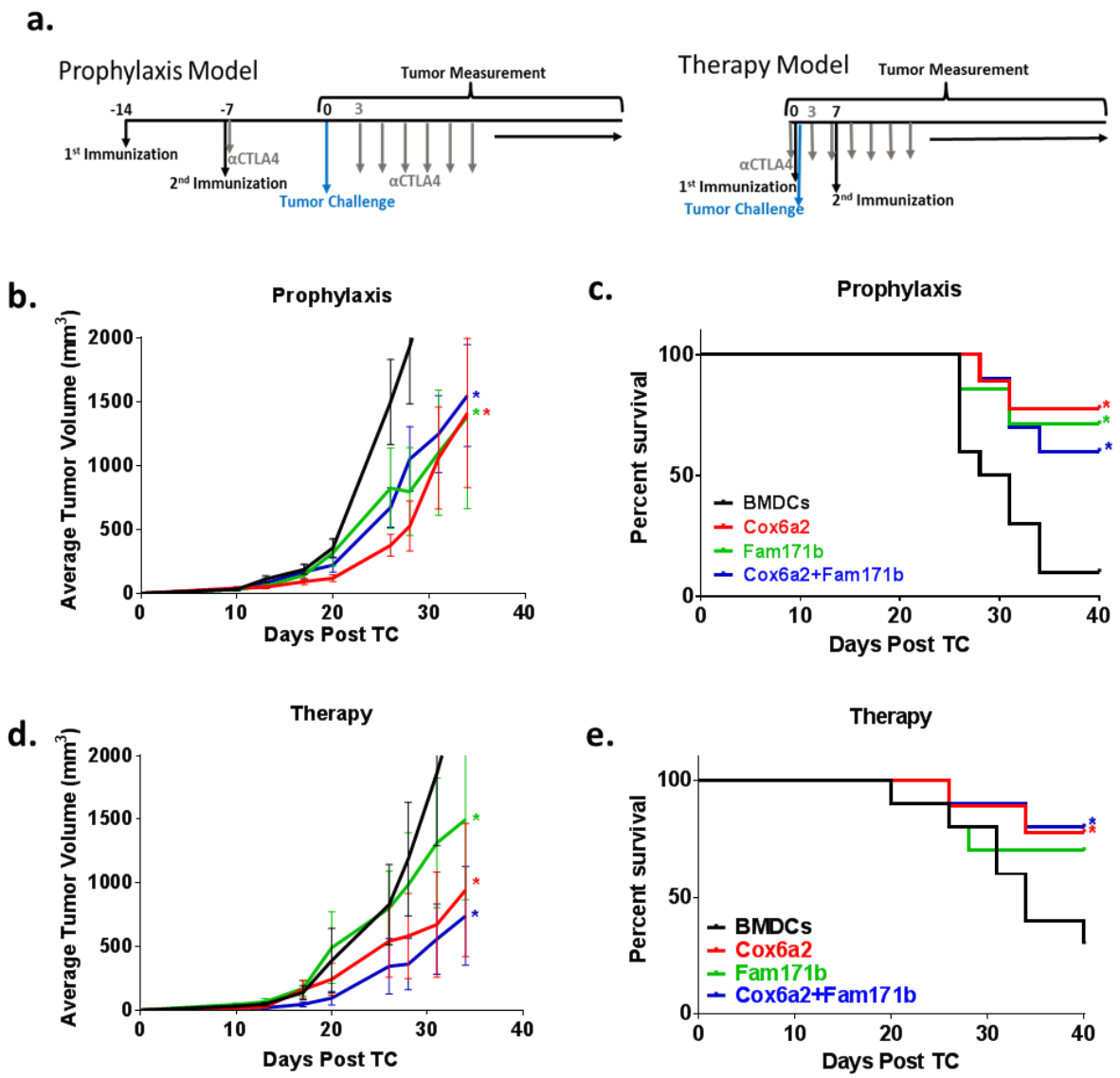


Figure 10 | Cox6a2 and Fam171b show significant tumor control in both prophylactic and therapeutic models
a. Experimental scheme: In the prophylactic model mice were immunized twice prior to tumor challenge. In the therapeutic model mice the vaccine was administer the same day as tumor challenge and were given boost one week later. **b,d.** The average tumor growth curves for BDMCs only compared to peptide(s) 261, 209 or the combination of 261 and 209. Means ± s.e.m. shown. **c,e.** Percent survival curves for BDMCs only compared to peptide(s) 261, 209, or the combination of 261 and 209. Statistical analysis was preformed using the log rank (Mantel-Coz) test. **(b-e)** n=10 mice/group **(b,d)** *p<0.05 (Student's t-test)

Anti-tumor activity of the best neoepitopes is CD8 dependent

Through using 21 amino acid long peptides for immunization, it is possible to stimulate both CD8 and CD4 T cell responses. It was unclear whether Cox6a2 and Fam171b neoepitopes were CD8 dependent, since no significant $CD8^+$IFNγ response was measureable against MC38-FABF immunized mice. To test whether the tumor immunity of our top neoepitopes are CD8 or CD4 dependent, mice were depleted of $CD8^+$ or $CD4^+$ cells through antibody depletion prior to immunizations and continued post tumor challenge (Figure 11a). Cox6a2 and Fam171b immunity was significantly lost when mice were depleted of $CD8^+$ cells, indicating both are CD8 dependent. Both groups that had been depleted of $CD4^+$ cells began to trend toward significant loss of tumor control in the late stage tumors (Figures 11b-c). This may be indicative of $CD4^+$ T cells being necessary for $CD8^+$ development of memory for long term responses, which will be investigated further.

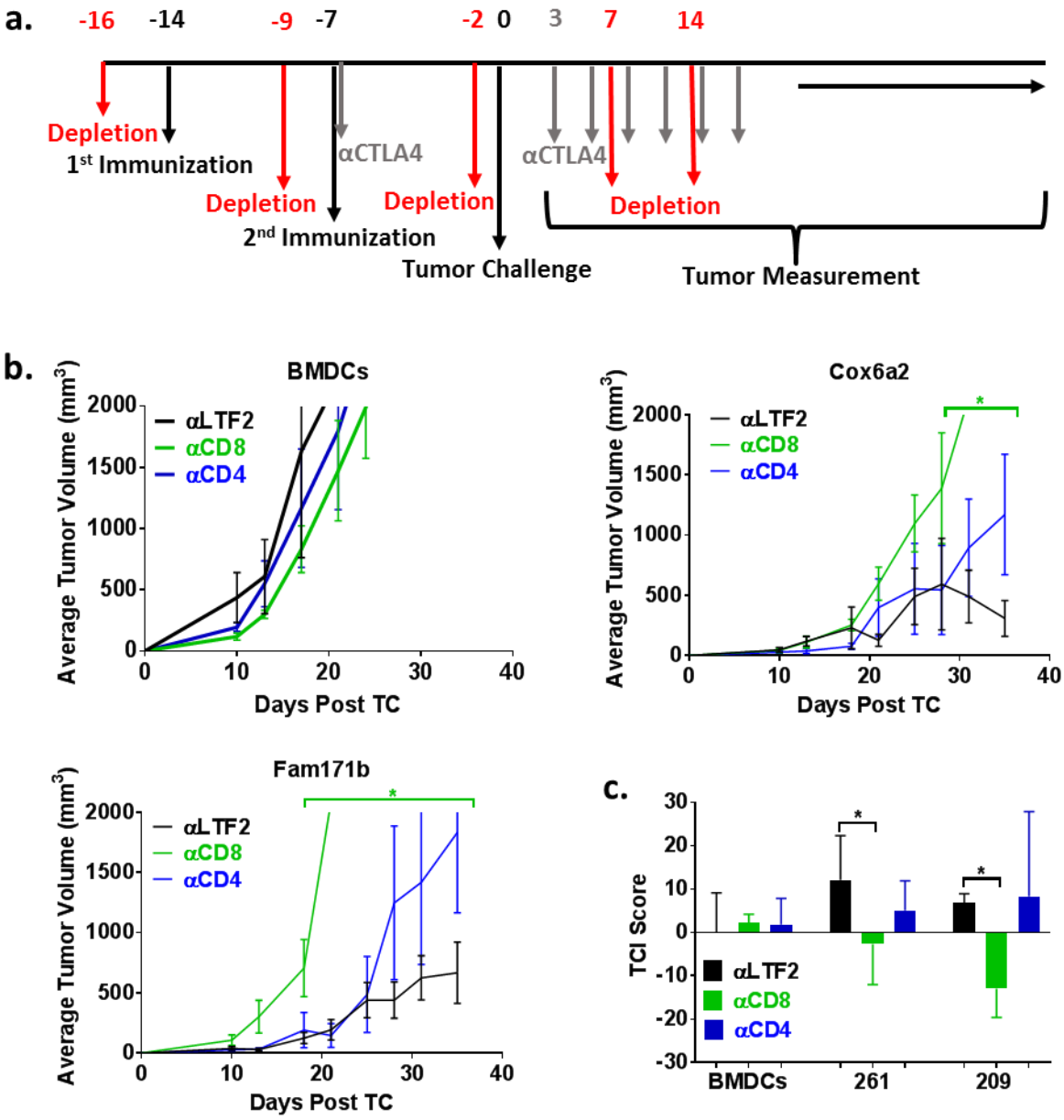


Figure 11 | Tumor control elicited by neoepitopes are CD8 dependent

a. Experimental scheme: Mice were immunized with neoepitopes 2 days after receiving 250µg of either αCD4, αCD8 depleting antibody or the isotype control αLTF2 antibody. 100µg of depletion antibodies were continued to be administered once every week until the mice were euthanized. **b.** The average tumor growth curves for BDMCs, peptide 261 and peptide 209 groups. Means ± s.e.m. shown. **c.** TCI scores are represented for the each of the vaccine groups and subdivided into their depletion groups. Means ± s.d. shown. **(b.c.)** n=10 mice/ group; *p<0.05 (Student's t-test).

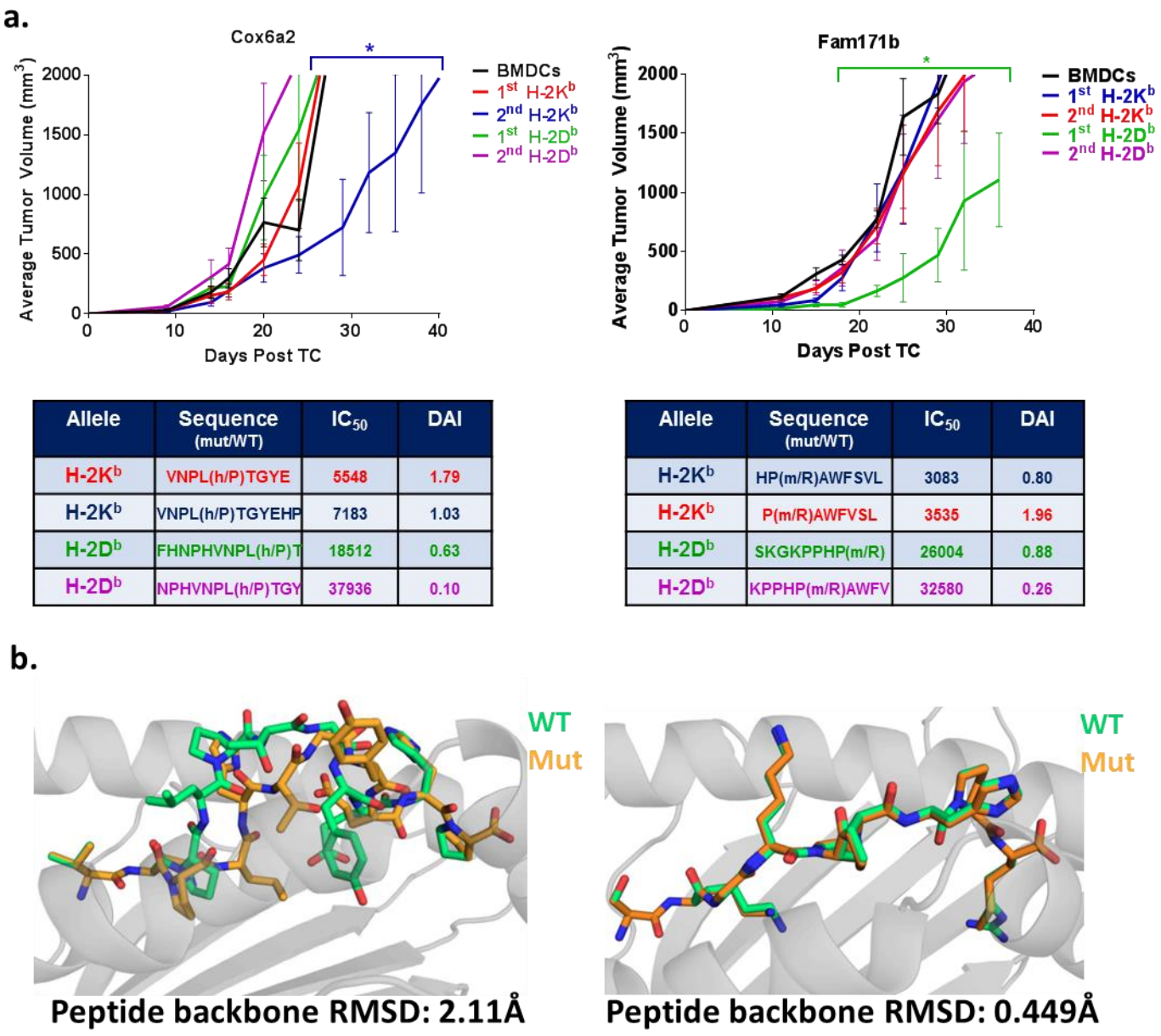
Cox6a2 and Fam171b precise peptides

The peptides used for immunization are processed and presented by the antigen presentation machinery, and the precise neoepitopes of the 21mer used for immunization was not known. In an attempt to accurately identify the precise epitope of Cox6a2 and Fam171b that is presented by the tumor and/or the BMDCs, I tested the top two precise peptides that have the highest predicted binding affinities to H-2K^b and H-2D^b. The predicted binding affinities of the epitopes are shown in the tables below Figure 12a. The second highest predicted epitope for H-2K^b of Cox6a2, labeled Kb2, and the highest predicted binder for H-2D^b of Fam171b, labeled Db1, consistently showed significant tumor control as compared to their other putative precise neoepitopes (Figure 12a). I conclude that these are the two major precise neoepitopes presented after processing and are responsible for the tumor responses.

Molecular modeling of MHC class I- peptide interaction

In order to understand the characteristics of these two neoepitopes that contribute to their immunological properties, extensive molecular modeling was conducted for the identified precise peptides and their predicted cognate MHC class I allele. Low-resolution centroid modeling was followed by high-resolution refinement and then repeated 10,000 times to find the highest likely conformation of the neoepitope and its wild type counterpart. PCA analysis was conducted on density-based spatial clustering of applications with noise (DBSCAN) to find the modeled populations with the favorable lowest energetic scores. The final conformations can be seen in Figure 12b. Cox6a2's neoepitope results in a completely different conformation than its wild type counterpart. Modeling suggests that replacing the p5 proline with a histidine allows the neoepitope to adopt a more compact backbone conformation in the binding groove, thus the

surface presented to a TCR differs considerably compared to the wild type. On the other hand, modeling Fam171b neoepitope behaved almost identical to the wild type peptide adopting the same conformation, but the neoepitope is presented more stably. This is similar to the conclusions drawn in the neoepitopes described in Duan et al. 2014[38], where a more ridged c terminus than its wild type counterpart results in a favorable neoepitope.



Allele	Sequence (mut/WT)	IC_{50}	DAI
H-2K^b	VNPL(h/P)TGYE	5548	1.79
H-2K^b	VNPL(h/P)TGYEHP	7183	1.03
H-2D^b	FHNPHVNPL(h/P)T	18512	0.63
H-2D^b	NPHVNPL(h/P)TGY	37936	0.10

Allele	Sequence (mut/WT)	IC_{50}	DAI
H-2K^b	HP(m/R)AWFSVL	3083	0.80
H-2K^b	P(m/R)AWFVSL	3535	1.96
H-2D^b	SKGKPPHP(m/R)	26004	0.88
H-2D^b	KPPHP(m/R)AWFV	32580	0.26

Figure 12 | Precise peptides for Cox6a2 and Fam171b

a. The two predicted highest binding affinity precise peptides for both H-2K^b and H-2D^b were used to immunize mice followed by tumor challenge. The upper figures show the average tumor growth curves for BDMCs only compared to the four precise peptides. Means ± s.e.m. shown. ; n=5 mice/group. *p<0.05 (Student's t-test). The lower tables represent the peptide sequence, the predicted IC_{50} scores (nM) for the stated MHC I, and DAI scores. All predicted using NetMHC4.0. **b.** Molecular modeling was performed for the precise neoepitopes that showed a significant tumor response. Low-resolution centroid modeling was followed by high-resolution refinement and then repeated 10,000 times to find the highest likely conformation of the neoepitope and its wild type counterpart. PCA analysis was conducted on density-based spatial clustering of applications with noise (DBSCAN) to find the modeled populations with the favorable lowest energetic scores. Differences were also quantified by superimposing average peptide conformations from the molecular dynamics simulations and computing RMSDs for all common atoms. Cox6a2 was modeled for H-2K^b (left) and Fam171b was modeled for H-2D^b (right).

Chapter 4

Plasticity of effector T cell phenotypes elicited by neoepitopes

Introduction

A major hurdle for cancer immunotherapies is overcoming the suppressive tumor environment and avoiding peripheral tolerization. Tumor specific T cells take on an exhausted phenotype, and recent efforts to overcome this exhaustion has been with checkpoint inhibitors, mainly αCTLA-4, αPD-1 and αPDL1. The idea behind these treatments is that by targeting the co-inhibitory molecules CTLA-4 and PD-1 found on T cells, the inhibitory signaling or "brakes" can be removed from these cells thus generating a stronger anti-tumor response[92,93]. Many studies have had positive outcomes and have demonstrated their efficacy[94–98]. However, these responses remain limited to only a subset of patients, which can differ significantly between tumor types. This complexity has been demonstrated not only in the significant disparities between different tumor types but further as the vast heterogeneity within a single patient's lesion(s)[89]. Numerous factors such as differences in tumor cell clonality, immune cell infiltration, angiogenic factors, mutational burden, etc. contribute to treatment response and ultimately determine treatment outcomes. The critical factor for selecting neoepitopes for therapy is to select neoepitopes that can avoid/overcome this peripheral tolerance and exhaustion.

In addition to immune checkpoint ligand expression, T cells that have become exhausted have distinct genetic patterns. Studies by Ahmed et al have identified molecular signatures of $CD8^+$ T cell exhaustion in chronic viral infections[126], and many of the same phenotypes are observed in tumor specific $CD8^+$ T cells. It is important to understand when tumor specific T cell exhaustion occurs, and specifically what phenotypes are more prone to suppression during tumor development. Work by Schietinger et al has demonstrated that tumor specific T cell dysfunction occurs early in tumor progression[127], and recent work by her group has characterized these T

cells that have a fixed chromatin state that are unable to be rescued[128]. These T cells can be broadly identified by the expression of $PD1^{hi}LAG3^{hi}CD38^{hi}CD101^{hi}$ on the cell surface. In contrast, they had also identified T cells within the tumor, $PD1^{hi}LAG3^{hi}CD38^{lo}CD101^{lo}$, that maintain a plastic reprogrammable phenotype. A major known driver of T cell exhaustion is the level T cell receptor (TCR) stimulation. It is well established that a high TCR avidity[129–131] and antigen concentrations[132,133] can drive an exhausted and dysfunctional phenotype. Through targeting neoepitopes that result in low avidity T cells and selecting the right antigen dose, it will elicit a robust effector T cell response within the tumor and avoid tolerization.

In the chapter of my thesis, I will discuss our preliminary experiments investigating which CD8 T cell response is ideal for a tumor rejection neoepitope. I hypothesize that through characterizing the neoepitope specific CD8 T cell response that results in tumor rejection, we can then pre-screen our neoepitopes to select the strongest candidates based on activated CD8 markers that we identify.

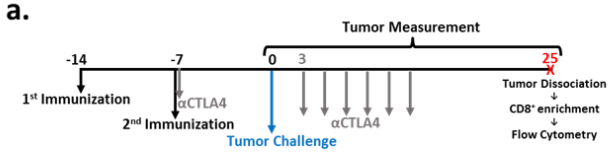
Results

Cox6a2 and Fam171b CD8$^+$ TILs are in a favorable plastic phenotypic state

In order to elucidate how these weak MHC class I binding neoepitopes are resulting in significant tumor control, I analyzed the tumor infiltrating lymphocytes (TILs) from the mice immunized with these neoepitopes (Figure 13a). I compare the neoepitope vaccines with BMDCs alone and peptide 244 (Figure 13b). Peptide 244 is predicted to bind MHC class I with a high affinity and had a significant CD8$^+$IFNγ response against MC38-FABF immunized mice, but showed no response in tumor control studies. Mice were immunized with BMDCs alone or one of the three peptides and then tumor challenged. On day 25 post tumor challenge, tumors were harvested and CD8$^+$ TIL were isolated. At this time point, Cox6a2 and Fam171b mice controlled the growth of the tumors, and the other groups' tumors had escaped (Figure 13c). Flow analysis on CD8$^+$ TILs isolated from tumors revealed that Cox6a2 and Fam171b immunized mice have significantly lower levels of CD38 expression on CD8$^+$PD-1^{high}LAG3^{high} cells (Figure 13d). This phenotype described by Philip et al. has a plastic and reprogrammable T cell state that is favorable for eliciting a robust tumor response[128]. Tumors from BMDC only and 244 mice showed high levels of CD38 on the same cell subset, which is indicative of an exhausted and fixed state of the T cells. Consistent with an overall lower exhausted state of the tumor rejecting neoepitope groups, TIM3 and 2B4 MFI is significantly lower than the controls[134] (Figures 13e-f). Indicative of a strong T effector response, the TIL of the neoepitopes had significantly lower levels of CD62L than the controls, hinting that they are likely more of an effector memory phenotype[135] (Figure 13g). Consistent with a plastic/reprogrammable phenotype, a key regulator of T cell differentiation, TCF1, was significantly higher for Cox6a2 and Fam171b, which corresponds with their lower CD38 expression[136] (Figure 13h). Based on

this data, I hypothesize that Fam171b and Cox6a2 result in an overall lower TCR stimulation, but strong enough to elicit an effector T cell response that avoids T cell exhaustion and dysfunction in the tumor microenvironment. Therefore, this allows for an effective neoepitope that elicits strong tumor response.

a.



b.

Peptide #	TCI Score	Gene Name	H-2K^b IC_{50}	H-2D^b IC_{50}	H-2K^b DAI	H-2D^b DAI	ELISpot
261	7.08	Cox6a2	8071.7	31233.9	1.01	0.13	-
209	6.21	Fam171b	3083	26004	1.96	0.88	++
244	2.17	Cd9	153.6	990.9	1.64	1.66	++++

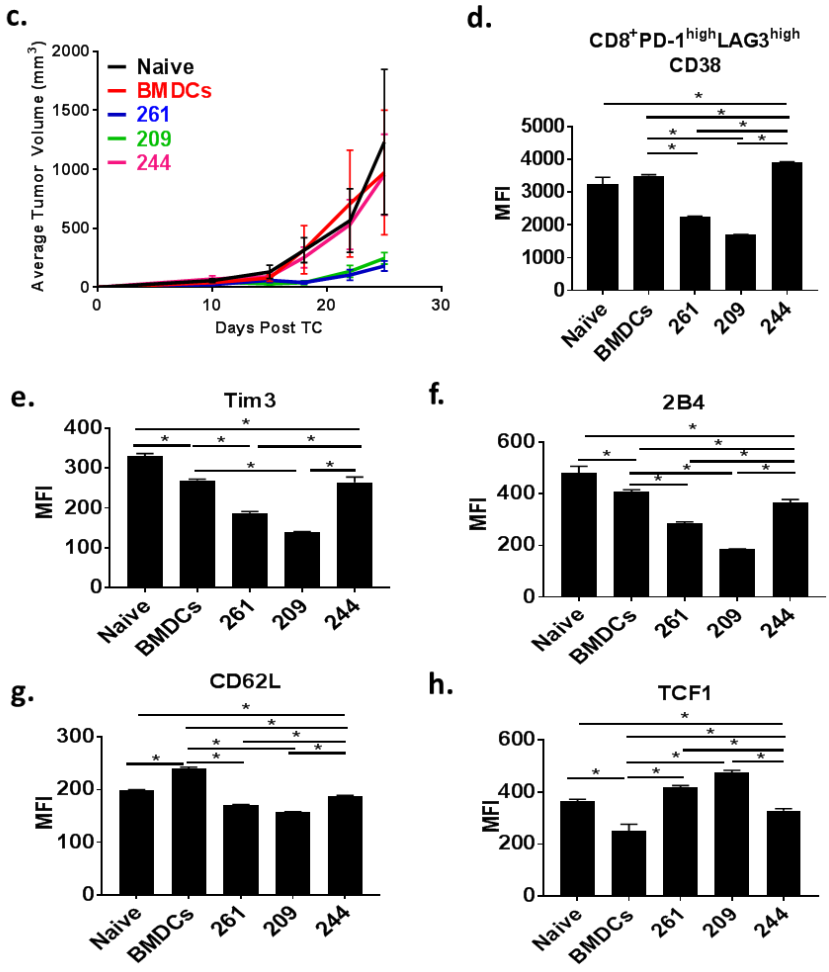


Figure 13 | CD8+ TIL from tumors of mice immunized with Cox6a2 and Fam171b have a more plastic and less exhausted phenotype
a. Experimental scheme. **b.** The table contains TCI scores, gene names, binding affinities, and immunogenicity measure via ELISpot for the three peptides used for immunizations where TILs were analyzed. Binding affinity scores were predicted using NetMHC4.0. ELISpot immunogenicity was determined from figure 8 where MC38-FABF immunized mice were tested for immune responses against all peptides. (≤0 spots/10^6 $CD8^+$ cells=-, 0-50 spots/10^6 $CD8^+$ cells=++, >140 spots/ 10^6 $CD8^+$ cells =++++). **c.** Average tumor growth curves for designated groups, with final measurement the same day as TIL harvest. Means ± s.e.m. shown. ; n=5 mice/group. **d-h.** Flow analysis of day 25 $CD8^+$ TIL from designated groups. Mean fluorescence intensity shown ± s.d.; n= 5 mice pre group, *p<0.05 (Student's t-test). **(d)** Gated on $CD8^+$ $PD\text{-}1^{high}$ $LAG3^{high}$. **(e-h)** Gated on $CD8^+$. This experiment has only been completed once, and is currently being repeated.

Chapter 5

Discussion

In this study, I identified nine neoepitopes out of 279 SNVs tested that were able to elicit significant tumor protection. It is important to note that not a single neoepitope(s) vaccine was capable of complete rejecting all tumors for a given group as well irradiated tumor cells. This may be indicative that neoepitopes may not be sufficient alone to reject aggressive solid tumors. Combination with other adjuvants, checkpoint inhibitors, or other treatments may be necessary to achieve complete protection. Ongoing studies are being conducted to enhance the tumor rejection in combination with other treatments and vaccine strategies, and to further investigate why vaccination with irradiated tumor cells provide complete protection.

Since I observed that peptide combinations can have varied effects compared to single peptides, it will be important to us to test the remaining 159 peptides from the groups that did not show a positive TCI score. Although, I believe our screening strategy did lead us to identifying the top nine neoepitopes for MC38-FABF, because the combination of the nine in one vaccine outperformed all other vaccines tested, including all 279 peptides in one vaccine. I have demonstrated the importance of the combination of multiple neoepitopes into a vaccine to elicit the most robust response. This will be important for natural tumors where heterogeneity and antigen escape play a major role in efficacy.

When measuring potential neoepitope efficacy and response in the clinical setting, the vast majority of studies have relied on the measurable T cell response *ex vivo*. Our study demonstrates that a measurable $CD8^+$ T cell response is not indicative to tumor control. Only two of our top nine neoepitopes had a significant $CD8^+IFN\gamma$ response measured via ELISpot, which is thought to be the most sensitive screening assay. I know that the tumor responses are CD8

dependent, because when I deplete $CD8^+$ cells from the mouse, I completely ablate protection of our top two neoepitopes Cox6a2 and Fam171b. The tumor response is not lost with $CD4^+$ depletion. This may be indicative that our T cell assays are not sensitive enough to measure neoepitope specific responses. This may be because neoepitope specific T cells that reject tumor successfully do not elicit as robust of a response as viral and model antigen T cells, which will be discussed further later on.

Most neoepitope selection pipelines use a 500 nM binding affinity threshold of an antigen to its cognate MHC class I, and only select the top binders for vaccination. In our study, only two of our nine neoepitopes have a predicted moderate and strong binding affinity, where only one is within the top 10% of predicted binders. The top performing neoepitopes are considered weak binders, and normally would be overlooked. This corresponds with the data from the Sahin et al trial where ~20% of immunogenic neoepitopes were predicted weak binders for MHC class I and II[123]. Predicted binding affinity of a neoepitope to its cognate MHC class I is not a predictor of tumor response. This may be indicative that our prediction algorithms are not accurate, but ongoing binding affinity measurements will clarify this discrepancy. Early indication that I was unable to synthesize tetramers (data not shown) hints that these truly are weak binders. I must identify further criteria beyond binding affinity predictions when selecting neoepitopes.

Our top two neoepitopes Cox6a2 and Fam171b were capable of significantly controlling tumor growth compared to their wild-type counterparts and prolong the survival of the mice. These neoepitopes were also demonstrated to be effective in a therapeutic model system. Further confirming that neoepitope combination is more beneficial, the combination of Cox6a2 and

Fam171b showed significant tumor control and especially in the therapy model where it outperformed the individual peptides in tumor growth control and survival. The precise peptides were identified for each, and both had predicted binding affinities >7000 nM for H-2K^{b} and H-2D^{b}. Both had a positive DAI score, confirming that the stronger mutant peptide than its wild-type counterpart is necessary for tumor protection.

Molecular modeling of Cox6a2 and Fam171b suggests two main conformation ideologies. Cox6a2's proline to histidine mutation at position five results in a completely different conformation of the peptide within the binding groove of MHC class I. The backbone mutation causes a favorable conformation that inverts the peptide in the groove, and exposes a hydrophobic residue to the TCR. This would increase the overall TCR avidity[137] and would be presented as a completely foreign antigen compared to the wild-type. Fam171b on the other hand has a c-terminus mutation that does not alter the conformation of the peptide in the binding groove. The arginine to methionine substitution at p9 is favorable to the binding of the c-terminus in the binding groove, and results in a more stable peptide than the wild type. This observation was made previously in the neoepitopes identified in Duan et al when the DAI predicted neoepitopes had a favorable rigid c-terminus[38].

When investigating CD8^{+} TIL from tumors of mice immunized with either the protective neoepitopes or a non-tumor protective high affinity immunogenic peptide, preliminary studies are consistent with the idea that the response the neoepitopes were inducing was favorable for tumor protection. Cox6a2 and Fam171b immunized mice have significantly lower levels of CD38 expression on CD8^{+}PD-1^{high}LAG3^{high} cells. This phenotype is indicative of a plastic and

reprogrammable T cell that has not undergone exhaustion. They also had lower levels of CD62L and higher levels of TCF1, which further confers with a plastic effector response[135,136]. The peptide that was unable to reject tumor had high levels of CD38 and exhaustive markers, such as TIM3 and 2B4. Even though this peptide elicited a robust T cell response *ex vivo*, it is likely that these T cells are quickly suppressed in the tumor microenvironment and become exhausted. Ongoing and future experiments will continue to examine the phenotype of the TIL from these mice and the other seven neoepitopes identified. I will also investigate the co-inhibitory markers identified in the recent study of Chihara et al, that studied the transcriptional regulation of dysfunctional T cells, and identified new markers of an exhaustive phenotype[138].

Taking all the data into consideration, I have identified mainly predicted weak affinity neoepitopes to MHC class I that cause significant tumor control and maintain a plastic effector phenotype within the tumor. I hypothesize that the overall avidity of the pMHC interaction with the TCR is the major factor that regulates this response. It has clearly been demonstrated that overstimulation of a high avidity T cells leads to an impaired response[129]. A study by Pace et al demonstrated that when mice were depleted of T regulatory (T_{reg}) cells, it allows for the expansion of low avidity effector T cells[139]. In our model systems, our mice are receiving αCTLA-4 which is known to deplete T_{reg} cells[140] in the tumor, which would already make the tumor environment preferential to the expansion of low avidity T cells based on the observations of Pace et al. A major contributor to the overall avidity, other than TCR affinity, is the peptide binding affinity to MHC. I hypothesize that a weak pMHC interaction can lead to an overall lower avidity T cell interaction, but strong enough overall avidity to generate an effector T cell

response. If I am able to clone out neoepitope specific T cells in our model, I can then begin to test this hypothesis.

In Figure 14, I demonstrate our working hypothesis for selecting the best candidate neoepitopes. The T cell repertoire is shaped via central tolerance and peripheral tolerance. T cells are deleted from the repertoire due to a high affinity for endogenous antigen or due to neglect. Of the remaining repertoire, if a T cell avidity is too high it leads to tolerization and exhaustion within the tumor environment; as observed with peptide 244. A high binding affinity of a peptide to MHC class I will likely result in an epitope that is stable and expressed in high levels on the cell surface. This high expression and stability would allow for an overall higher avidity with the T cells. Because I see a strong immune response *ex vivo,* but no tumor response and an exhausted phenotype to these antigens, I believe the high avidity interactions are being tolerized rapidly within the tumor. I hypothesize that through identifying the neoepitopes that contribute to an overall lower avidity but strong enough to elicit a response, we can begin to understand what makes a strong candidate neoepitope. I believe that the pMHC affinity is a major contributor to overall avidity, and can be used for selecting neoepitopes. This is supported by the observation that the neoepitopes I identified in this study mainly have a weak to moderate binding affinity and a positive DAI.

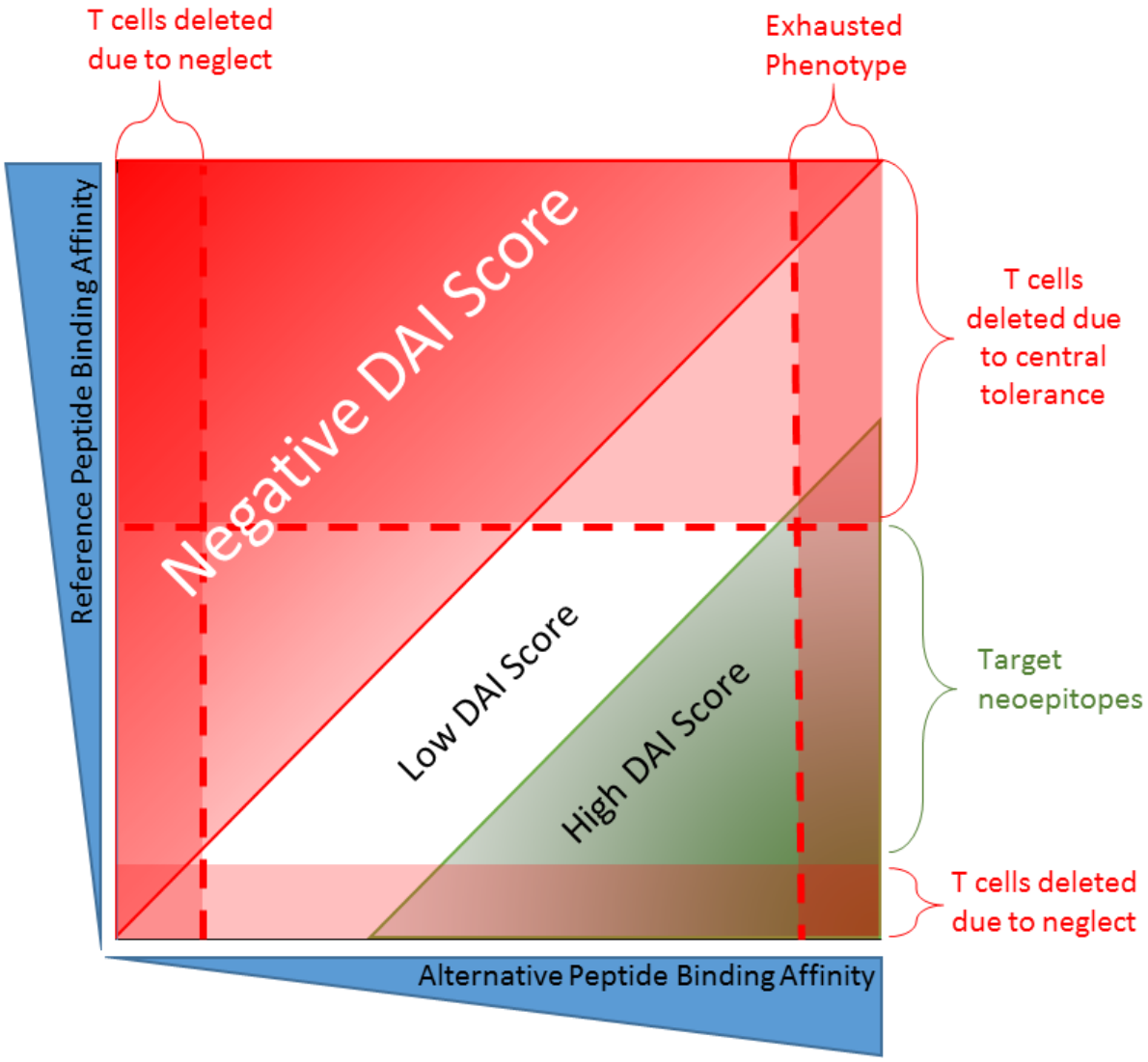


Figure 14 | Working theory for targeting tumor rejecting neoepitopes
This figure represent the working hypothesis for selecting the best neoepitopes for personalized cancer immunotherapies.

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